CONDITIONING FACTORS IN THE RELATIONSHIP BETWEEN STRESS AND OPIOID SELF-ADMINISTRATION IN RATS

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ABSTRACT

TITLE OF DISSERTATION: "Conditioning Factors in the Relationship between Stress and Opioid Self-Administration in Rats"

YAVIN SHAHAM, DOCTOR OF PHILOSOPHY, 1992

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The present experiments examined the effect of stress on oral opioid (morphine and fentanyl) self-administration (SA) and withdrawal, and further examined the role of conditioning factors in the relationship between stress and opioids in rats. In Experiment 1, male rats were exposed to four different conditions for each of the two drugs: *Paired-stress* groups were exposed to 15 min/day of immobilization (IM) stressor that reliably predicted the opioid SA period for 6 h/day in the home cage; *Partial-Conditioning* groups were exposed to IM stressor that predicted the drug SA period in 50% of the days; *Conditioned-Inhibition* groups were exposed to IM stressor that predicted the absence of the drug; and *Control* groups were not exposed to the stressor. The *Paired-stress* and the *Partial-Conditioning* groups increased their drug SA and manifested a more severe withdrawal syndrome compared with the *Conditioned-Inhibition* and *Control* groups. The experimental animals were further examined during a 30-day relapse phase conducted after 60 days of drug SA followed by 3 weeks of a drug-free, stress-free period. The original four experimental groups within each drug class were divided into two conditions. The *stress* groups were exposed to the IM stressor prior to the opioid SA period; and the *Control* groups were not exposed to the stressor. The *stress* groups increased their drug SA and manifested a more severe withdrawal syndrome compared with the *Control* groups. In Experiment 2, rats were trained to lever press for fentanyl solutions in operant conditioning chambers. Rate of responding for the drug after exposure to electric footshock and without exposure to the stressor was the main measure in this experiment. Animals increased their fentanyl SA during the stress condition as compared to a non-

stress condition.

These experiments indicate that exposure to stress increases opioid SA in rats; that stress enhances the opioid withdrawal syndrome; and that conditioning factors, or the temporal relationship between exposure to stress and the opioid availability, partially mediates the effect of stress on opioid SA and opioid withdrawal. The implications of the study results to mechanisms underlying the stress-opioid interaction, future research directions, and clinical implications are discussed.

Conditioning Factors in the Relationship between Stress and Opioid Self-Administration in Rats

By

Yavin Shaham

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Opioid[1] abuse, currently affecting 400,000-600,000 people in the United States, is a severe social and psychological problem associated with deterioration in social functioning and health status, and with a high rate of psychopathology (Hollister, 1989; Jaffe, 1985). Further, despite all legal and educational efforts, it was estimated that Americans in 1990 spent $12.3 billion to purchase illegal heroin alone (Washington Post, June 20, 1991).

Epidemiological data suggest that stress is positively related to use and abuse of opioids and other addictive drugs (see Shiffman & Wills, 1985). The high co-morbidity of substance abuse and Post-Traumatic Stress Disorder (Keane & Wolfe, 1990) is another illustration of the positive relationship between drug abuse[2] and stress. At present, however, although many ideas have been suggested, the mechanisms underlying the relationship between stress and opioid abuse as well as other drugs of abuse are not clear (Grunberg & Baum, 1985). This situation is not surprising because few experiments (Dib, 1982; Dib & Duclaux, 1985; Shaham et al., 1992) have examined the effect of stress on opioid consumption or use in controlled experimental settings using random assignment and parametric manipulation of the key independent variables (stress levels and drug administration).

The purpose of the present experiments was to examine the effect of stress on opioid self-administration (SA) in controlled experimental settings using rats and to examine the involvement of conditioning factors in the relationship between stress and SA of opioids. In the sections below a background for the

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[1] An opioid agonist is defined as any compound, natural and synthetic, with morphine-like actions, whereas an opioid antagonist is a compound which blocks morphine-like drug actions (Jaffe & Martin, 1990). The term "opiate," once used to designate drugs derived from opium (Jaffe & Martin, 1990), is not used in the present paper.

[2] Drug abuse refers to the use, usually by self-administration, of any drug in a manner that deviates from the approved medical or social patterns within a give culture (Jaffe, 1985). In the present paper, opioid abuse refers to the consumption of illicit opioids by humans (e.g., use of illegal heroin). Opioid use refers to the consumption of opioid drugs in an approved medical setting (e.g., consumption of methadone in drug rehabilitation center).

hypotheses is provided. This background information includes a discussion of stress, a description of the opioid drugs, drug addiction concepts, epidemiological and clinical data on the relationship between stress and opioid abuse, effects of opioid drugs on the stress response, learning factors in drug abuse, and other pertinent information.

The stress concept

Systematic research concerning the effects of stress on the body can be traced to the work of Walter Cannon in the early 20th century (Cannon, 1935). Cannon defined stress in terms of the profile of emotional and physiological responses to danger and argued that stress causes a disruption of the body's homeostasis. He further delineated the role of the sympathetic nervous system and adrenal medulla in the response to stress. Cannon also viewed stress as a potential cause for medical problems and suggested that emotional stress may cause physiological disturbances. Selye, in his biological model of stress, argued that exposure to any stressor (e.g., heat, cold, pain, X-rays) elicits a characteristic response (i.e., the stress response) consisting of enlargement of adrenal glands, shrinkage of the thymus gland, and bleeding ulcer (Selye, 1956). Selye further pointed out the important role of the adrenal cortical glucocorticoids in mediating the stress-induced physiological changes, and tried to link stress to subsequent diseases (e.g., cardiovascular disease, arthritis, and kidney diseases) in his model of the General Adaptation Syndrome (GAS). Specifically, the GAS consists of three phases: First, the "alarm reaction" in which the organism become aware of the stressor and prepares to resist it by increasing adrenal activity, and cardiovascular and respiratory functions. Second, the organism enters a "stage of resistance" in which various coping mechanisms are applied in order to deal with the stressor and typically a suitable adaptation is achieved. Third, when the bodily reactions to the stressor(s) are prolonged, the organism may be placed at risk for irreversible physiological damage due to a depletion of the adaptive reserves, an "exhaustion phase." That is, Selye argued that after prolonged exposure to stress, the adrenals may become exhausted, secretion of corticosteroids then decreases

rapidly, and the organism is at risk for organ damage and disease. Lazarus (1966) developed a psychological model of stress in which he postulated that the physiological stress response is entirely dependent on the subjective appraisal of the stressor and not on the stressor *per se*. He argued that unless a person perceives a situation as threatening, he/she would not experience stress. Specifically, once a potential stressor is encountered, the event is perceived as either threating, harmful, challenging or benign. Based on this initial appraisal, a given event may mobilize different behavioral and psychological coping processes that determine the individual's physiological and psychological reaction to a given stressor. Mason (1975) further suggested that psychological factors such as predictability over the stressor may mediate the stress response. In a series of studies carried out by Mason and others (see Cohen, 1980; Glass & Singer, 1972; Mason, 1975), it was found that in both humans and animals, different patterns of physiological and psychophysiological responses were elicited by the same stimulus or event if psychological parameters such as predictability, controllability, perceived controllability and uncertainty were experimentally manipulated.

Currently, stress is viewed as a process in which environmental or psychological events, called stressors, come to threaten the organism's safety and well-being (Baum, Singer, & Baum, 1981). The stress process consists of three distinctive, but related elements, that include the stressors, the stress response, and intervening factors or processes that mediate the effect of stressors on the organism (Baum, 1990; Baum, Singer, & Baum, 1981; Cohen et al., 1982). Stressors are any event, perceived or real, that profoundly interfere with the organism's normal steady state. These steady state disruptions comprise the stress response and can be manifested at either the physiological (e.g., increased corticosteroid or catecholamine secretions and blood pressure elevations), psychological (e.g., anxiety and depression), or behavioral (e.g., performance deficits) levels (Baum, 1990; Baum et al., 1987; Cohen et al., 1986). Intervening variables include factors such as personality, personal resources, genetic predispositions, cognitive appraisal, coping mechanisms, and social

support (Cohen et al., 1982). The definition of stress is further complicated because there exist only modest correlations among the manifestations of the stress response at the physiological, behavioral, and psychological levels (Baum, Grunberg, & Singer, 1982; Glass & Singer, 1972). In addition, it seems that the classification of acute/chronic stress based on the stressor duration commonly found in the animal literature is too simplistic. For example, acute exposure to stress may result, at times, in long-term negative consequences as in the case of exposure to trauma. Evidence from human studies further indicate that the persistence of the stress response may be affected not only by the duration of exposure to a given stressor, but also by other factors such as the appraisal of threat, coping mechanisms, conditioning factors, and intrusive thoughts (Baum, 1990; Baum, O'Keeffe, & Davidson, 1990).

Exogenous and endogenous opioids

Opioids are a class of compounds consisting of both the natural opium alkaloids, of which morphine is the prototype, and related semisynthetic (e.g., heroin), and synthetic (e.g., methadone and fentanyl) drugs. The endogenous opioid peptides, enkephalins, endorphins, and dynorphins also belong to the opioid class (Jaffe & Martin, 1990). Opioid drugs are used clinically for alleviating pain, suppressing the cough reflex, and to provide symptomatic relief of diarrhea (Jaffe & Martin, 1990; Cox, 1990). Other actions of opioids include, among others, respiratory depression, endocrinological changes, immunosuppression, decreased sex drive, decreased aggression, sedation, drowsiness, decreased ability to concentrate, and mood changes (Jaffe, 1985; Jaffe & Martin, 1990).

The present research utilized two opioid agonists, morphine and fentanyl. The chemical structure of these drugs is provided in Figure 1. Morphine, the prototype opioid agonist, was first extracted from the opium poppy in 1803 in Germany and named after Morpheus, the greek god of dreams (Way & Way, 1989). Morphine is a small molecule (molecular weight of 285.3), with pKa=9.85 and it is highly water soluble in the form used in this study (morphine-sulfate, 1 g dissolved in 15.5 ml of water at 25^0 Celsius). Morphine is well absorbed from

subcutaneous and intramuscular sites as well as from the mucosal surfaces of the nose and gastrointestinal tract. By the oral route, however, morphine levels may be considerably reduced (up to 75% in humans) because of a significant first-pass metabolism in the liver. Morphine, which contains free hydroxyl groups, is mainly conjugated with glucuronic acid and eliminated by glomerular filtration mainly in the form of morphine-3-glucuronide. Morphine's half-life is approximately 1.9 h (Jaffe & Martin, 1990; Way & Way, 1989; Windholz et al., 1983).

Fentanyl, a synthetic opioid drug about 100 times more potent than morphine as an analgesic (i.e., 0.1 mg/kg of fentanyl has the same analgesic effect as 10 mg/kg of morphine), was first synthesized by Paul Janssen in Belgium in the early 1960s. Fentanyl is also a small molecule (molecular weight of 336.5) with a pKa=7.7 and it is highly water soluble in the form used in this study (1 g of fentanyl-HCl dissolved in 40 ml of water at 25^0 Celsius). Fentanyl is a prototype drug of the fentanyls group that contains over 200 fentanyl analogs (e.g., sufnetanil, alfentanil and 3-methyl-fentanyl). The fentanyls were introduced into the United States in 1970s and today it is estimated that they are used for pain control and anaesthesia in over 70% of all surgical procedures (Henderson, 1990). The high lipid solubility of the fentanyls ensure that these drugs will reach the brain very rapidly (a few minutes) regardless of the route of administration. Brain levels of fentanyl after intravenous administration are 10 times higher than that in plasma. By comparison, morphine brain levels are only 1/20 of plasma concentrations. The main metabolic route of fentanyl is oxidative N-dealkylation and it is eliminated primarily in the urine as polar metabolites (e.g., norfentanyl). In humans, fentanyl reaches its peak analgesic effectiveness within 4 min of intravenous injection, and the terminal elimination half-life of fentanyl is approximately 3.7 h (Henderson, 1990; Hibbs, Perper & Winek, 1991; Jaffe & Martin, 1990; Windholz et al., 1983). Fentanyl is self-administered by laboratory animals, and experienced opioid users perceive fentanyl as qualitatively similar to heroin (Lal et al., 1977; Henderson, 1990). In 1979, the fentanyls were first introduced in California as illicit drugs under

the names of "China White" or "synthetic heroin." In the last decade these drugs were responsible for over 100 deaths in the U.S., mainly due to respiratory depression, and abuse of fentanyl has become a problem among health professionals. For example, according to one survey it is the most popular substance abused by anesthesiologists (Henderson, 1988). The potency of the fentanyls, the fact that most fentanyl analogs are pharmacologically active, and the increasing legal efforts to curtail imported heroin suggests that the use of the fentanyls as illicit drugs may increase in the future (Henderson, 1988).

The endogenous opioid peptides and receptors are widely distributed throughout the nervous system (e.g., spinal cord, mesolimbic structures and cortex), the neuroendocrine system (e.g., the pituitary and adrenal medulla), the gastrointestinal tract, and several other structures (Akil et al., 1984). The localization of these peptides in specific neural pathways and endocrine tissues has led to the concept that opioid peptides normally function as neurotransmitters, neuromodulators, and hormones (Cox, 1990). The opioid peptides are thought to be involved in the stress response, analgesia, rewarding drug effects, hormonal regulation, mood, and learning and memory (Akil et al., 1984; Akil & Watson, 1991; Jaffe & Martin, 1990).

Subtypes of opioid receptors have been defined on the basis of the differential binding affinity of various opioid agonists and antagonists, and related compounds. Drugs similar to morphine and some synthetic opioids preferentially bind to and produce effects at *mu* receptors. The *kappa* receptor, named for the prototypic drug ketocyclazocine, is thought to mediate the physiological actions of dynorphin peptides and opioids such as pentazocine and butorphanol. *Delta* receptors appear to be the preferential binding site for the met-enkephalin and leu-enkephalin endogenous opioid peptides, and *epsilon* receptors are thought to mediate some of the effects of beta-endorphin. The *sigma* receptor, an atypical opioid receptor devoid of analgesic action and named for the drug SKF 10,047, is one of the binding sites for phencyclidine (Akil et al., 1984; Jaffe, 1985; Cox, 1990). However, it should be noted that most endogenous and exogenous opioids bind to more than one receptor subtype, and

additional receptor sub-types, e.g., *lambda*, are thought to exist. Further, even within each receptor subtype, more than one affinity state may exist (Akil & Watson, 1991; Jaffe, 1985; Werling, Puttfarcken & Cox, 1988). At the cellular level, opioid effects are mediated via the cAMP second-messenger system. In this system, the interaction of an opioid agonist or partial agonist with a receptor stimulates guanine nucleotide-binding protein (Gi) that inhibits the enzyme adenylate cyclase, an enzyme leading to the production of cAMP (Jaffe & Martin, 1990).

Chronic opioid agonist use is associated with tolerance[3], cross-tolerance[4], drug dependence[5] (physical and psychological), cross-dependence[6], and a withdrawal syndrome after the discontinuation of opioid administration. In humans physically dependent on heroin or morphine, a severe withdrawal syndrome usually lasts for 7-10 days after discontinuation. This syndrome is manifested by hyperactivity of the sympathetic nervous system, restlessness, drug craving, yawning, runny nose, chills, fever, loss of appetite, insomnia, hypertension, aches and pain, anxiety and dysphoria (Cox, 1990; Jaffe, 1985). More long-term effects of opioid withdrawal include disturbed sleep, poor self-image, and drug

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[3] Drug tolerance is a state of decreased responsiveness to the pharmacologic effect of a drug as a result of prior exposure to that drug. Drug tolerance is manifested as a shift in the dose-response function to the right (Cox, 1990). Drug tolerance can be due to increased disposition of a drug after chronic use (pharmacokinetic or metabolic tolerance); compensatory changes in receptors, enzymes, or membrane actions (pharmacodynamic or functional tolerance); or the organism's ability to compensate for the behavioral drug's effects (behavioral tolerance) (Hollister, 1989).

[4] Cross-tolerance between drugs refers to the phenomenon where exposure to one drug produces tolerance to it and also to the other drug (Cox, 1990).

[5] Drug dependence is a state in which the organism requires the presence of a drug for normal functioning. Psychological dependence is characterized by compulsive drug-seeking behavior in order to obtain the drug. Physical dependence occurs when the termination of drug administration results in pathophysiologic disturbances known as a withdrawal syndrome. This syndrome is alleviated by readministration of the drug. It is thought that physical dependence occurs because the body adjusts to a new level of homeostasis during the period of drug use and reacts in the opposite direction when the new equilibrium is disturbed (Hollister, 1989; Cox, 1990).

[6] Cross-dependence refers to a state in which the dependent state produced by one drug can be maintained by another drug after the discontinuation of the first drug (Cox, 1990).

craving (Cox, 1990; Jaffe, 1985). Other opioid agonists produce a similar type of physical dependence but the duration and profile of withdrawal symptoms vary with the agonists' bioavailability and receptor action (Cox, 1990; Jaffe, 1985).

Effect of stress on opioid use in humans

In the last three decades numerous studies have been conducted to examine the effects of stress on initiation, maintenance, and relapse to drug and alcohol abuse. Though not specifically stated in every study presented, the theoretical framework guiding most of these studies was that drug abuse or use may serve as a coping mechanism in order to buffer the aversive aspects of exposure to stress, "the stress-reduction/buffering hypothesis" (Alexander & Hadaway, 1982; Cohen et al., 1985; Wills & Shiffman, 1985). For example, according to Alexander and Hadaway (1982, p.367) "opiate addiction is an attempt to adapt to chronic distress of any sort through habitual use of opiate drugs."

Epidemiological data, in general, tend to support the notion that stressful life events are positively[7] correlated with the initiation of drug abuse among adolescents (Bruns & Geist, 1984; Timmer, Veroff & Colten, 1985; Wills, 1986). However, most epidemiological studies have only evaluated the relationship of stress and drug abuse for cigarette smoking and alcohol abuse, not opioid abuse. Further, drug abuse data in these studies are based upon self-reports that may not be reliable measures for detecting illegal drug abuse because drug addicts are not likely to reveal their illegal activity. Therefore, examination of the relationship between stress and opioid abuse is limited to studies that have directly measured illicit opioid by objective measures (i.e., detection of drugs in urine samples). Table 1 summarizes studies that examined the relationship between stress and opioid use in opioid addicts consuming illicit or legal opioid agonists (i.e., methadone).

Inspection of Table 1 reveals that 12 of the 14 studies reported a positive association between stress and opioid use. These studies suggest that heroin is

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[7] Some epidemiological studies have failed to find a positive relationship between stress and adolescent drug use. For example, Swaim et al. (1989) found that emotional distress could account for only 4.8% of the variance in drug use in a sample of 563 adolescents.

used to alleviate stress (Khanatzian et al., 1974). Further, stressful life events are related to the precipitation of withdrawal symptoms (Whitehead, 1974), the initiation of opioid use (Rounsaville et al., 1982), continuation of opioid use (Grey et al., 1986; O'Doherty, 1991; Prusoff et al., 1977) and relapse to drug use (Chaney et al., 1982; Kosten et al., 1983, 1986; Krueger, 1981). Also, higher dosages of methadone are negatively related to interpersonal stress (Robbins, 1977).

Conclusions based on these studies, however, are compromised by several methodological and conceptual limitations. Methodological limitations include small sample sizes, failure to use validated questionnaires, inadequate control groups and short durations of assessment (see Table 1). Further, even adequate epidemiological studies cannot address the issue of causality. With other drugs (e.g., cigarette smoking), a causal relationship between stress and increased drug abuse has been established experimentally by using random assignments of subjects to groups exposed to different levels of stress and then measuring drug consumption (Schachter, 1978). Although this experimental design may not be possible to employ with illegal opioids due to ethical considerations, it may be appropriate in methadone clinics where the drug is given on a medical basis.

Another limitation of the studies in Table 1 is related to the measurement of stress. As mentioned before, stress is defined as a process in which subjective appraisal of environmental events may lead to a stress response at either the physiological, psychological, or behavioral level (Baum et al., 1981). None of the studies reviewed measured any physiological aspects of the stress response. Although some of them assessed psychological consequences, most used retrospective self-reports of life events using a questionnaire originally developed by Holmes and Rahe (1967). The retrospective assessment of life-events can be criticized on several grounds. These problems include faulty memory and cognitive biases (e.g., people tend to overestimate the aversiveness of events in their past and use them as an excuse for current drug use) (Hall et al., 1990; O'Doherty & Davies, 1987). Despite these limitations, the fact that most of the

published studies examined found a positive relationship between stress and opioid use suggests that stress is related to opioid use and abuse.

Effect of stress on opioid self-administration in animals

In the animal literature, numerous studies have explored the interaction between endogenous and exogenous opioids and stress. However, the majority of studies are not directly related to the question of whether stress increases opioid use because the ingestion of the drug was not under the animals' control. The positive reinforcing properties of opioid drugs as well as other drugs can be assessed using a self-administration (SA) paradigm. The basic premise of this methodology is that psychoactive drugs, like many other stimulus events (e.g., food and water), can control behavior by functioning as positive reinforcers (Brady, 1991; Johanson, Woolverton & Schuster, 1987). In the drug self-administration paradigm, the drug's administration is under the animal's control and in this way an objective measure of drug-seeking behavior can be obtained. The main dependent variable employed in this paradigm is rate of operant responding (e.g., lever pressing) resulting in drug administration. The positive reinforcing properties of drugs under the SA paradigm also can be measured by utilizing a choice procedure in which the drug and vehicle (e.g., water) are concurrently available. In this situation the main dependent variable is the proportion of drug consumed (Carroll & Meisch, 1979; Johanson, 1989; Stolerman & Kumar, 1970). Opioid agonists are readily self-administered by many species (e.g., rats, mice and monkeys) under the self-administration paradigm indicating that they can serve as positive reinforcers (Griffiths, Bigelow & Henningfield, 1980). Further, the remarkable concordance between drugs self-administered by animals and drugs abused by humans makes the SA paradigm an important experimental tool in the examination of pharmacological and behavioral factors of drug abuse (Brady, 1991).

Two rat SA studies designed to evaluate the relationship between stress and morphine SA have been reported. Dib and Duclaux (1982) trained rats to self-administer intracerebroventricular morphine (0.5 ug/ul) under a fixed-ratio-1 (FR-1 schedule of reinforcement in which every lever press resulted in a drug

administration). After the training phase, the rats self-administered the drug for 1 h/day in the operant chamber. During this 1 h period, the rats were exposed to 15 min of intermittent electric footshock stressor. Results indicated that during the stress period, the animals significantly increased morphine SA in comparison to the non-stressful periods. Dib (1985) further reported an increased intrathecal morphine (1 ug/ul) SA during exposure to electric footshock stressor in rats using the Dib and Duclaux (1982) procedure. In these studies, however, the increased morphine SA was only observed *during* exposure to the noxious electric footshock stressor, but not prior to or after the stressor administration. Therefore, an alternative explanation for these results may be that the rats learned to increase their morphine SA in order to decrease the pain induced by the footshock. In other words, based on these studies the stress-induced morphine SA may not be related to stress-induced changes in the reinforcement efficacy of opioids, but instead to the noxious properties of the electric footshock.

In the last year we have conducted several studies using two animal paradigms in order to examine whether immobilization (IM) stressor increases oral morphine and fentanyl consumption (Shaham, et al., 1992). The design of these studies circumvents the confounds of Dib's studies because the stressor used (immobilization) is not a painful stimulus, and the drug SA period followed the stressor administration. We have found that rats self-administer opioid drugs by drinking solutions that are provided in home cages and in operant chambers. In one model, animals were given access to morphine (0.25-0.5 mg drug/ml tap water) or fentanyl (5-20 ug drug/ml tap water) for seven hours per day in home cages. Every fifth day, the animals were given a choice between the opioid solution and water (a modification of a procedure reported by Stolerman & Kumar, 1970). A total of 5 choice days were conducted in this study. A 15 min/day IM stressor was administered prior to the drinking sessions. This stressor has been found to cause a reliable elevation in plasma corticosterone levels (Kant et al., 1987). A main effect of stress across time was observed under these conditions. Specifically, animals exposed to the stressor showed greater drug preference for

opioids compared to controls (see Figure 2a). No significant differences among the groups were observed during the forced consumption days indicating that stress did not affect fluid consumption *per se*. Therefore, it is unlikely that the increased drug preference during choice days is related to effects of stress on overall fluid consumption. In addition, we examined the effect of stress on sub-groups of morphine animals (total n=9) for a longer duration of time (i.e., 11 choice days). In the last choice day, animals exposed to IM stressor showed 70% preference for morphine in comparison to 34% preference in controls (see Figure 2b).

We further found that animals in this paradigm developed physical dependence (i.e., 1 mg/kg of naloxone s.c. produced measurable withdrawal symptoms such as teeth chattering, loss of body weight, and diarrhea). Also, plasma corticosterone levels in the stress groups were significantly higher than in controls indicating that the stress manipulation was effective. Finally, in a different group of animals, over time, IM stressor did not affect preference for a quinine solution (0.3 mg/ml) that is of similar bitterness to the morphine solution. Therefore, the increased preference to the opioid solution in the stress groups during choice days was not a result of the effect of stress on taste sensitivity or taste preference.

In a second animal model, rats were given one hour a day access to fentanyl solutions in operant chambers. Drug SA was induced by a food schedule of 1 h per day of food access prior and during the session in the operant chambers (a procedure modified from Carroll & Meisch, 1979). In this set-up, animals had to press a lever in order to have access to fentanyl (5-50 ug/ml). Preliminary data indicate that in the first three weeks of access to the drugs, rats given ad libitum access to water in their home cage consumed larger amounts of fentanyl in the operant chamber after exposure to 15 minutes of IM each day, compared to control animals. However, no significant differences between the groups were observed when animals were given a choice between the fentanyl solution and water every forth day, and during forced consumption days in the next six weeks of this experiment, indicating that the stress manipulation in this paradigm did not

affect oral opioid SA.

This preliminary work indicates that stress can reliably increase preference to opioid solutions in the home-cage paradigm. In contrast, based on our previous operant study, no firm conclusion can be drawn concerning the effect of stress on fentanyl SA. A plausible reason for the lack of effect of stress on fentanyl consumption in the operant model may be related to the food schedule in this experiment (1 h per day). Body weight data indicated that the animals were food-deprived. Because food-deprivation is known to increase drug SA (Meisch & Carroll, 1987), the lack of difference in drug consumption between the control and stress groups may have resulted from the chronic food-deprivation effects on drug consumption that masked the effect of stress. Further, it may be that the between-subjects design employed in this experiment masked the effect of stress due to large individual differences in operant responding to fentanyl.

Two other lines of evidence from the animal literature are related to the relationship between stress and drug SA. It has been reported that in rats, stressors, such as uncontrollable footshock, IM, and tail-pinch increase SA of ethanol and amphetamines (see Pohorecky, 1990 and Piazza et al., 1991a for reviews). In addition, social isolation (Alexander, Coambs & Hadaway, 1978; Bozarth, Murray & Wise, 1989; Hadaway et al., 1979; Marks-Kaufman & Lewis, 1984) and food deprivation (see Carroll & Meisch, 1984, for a review) result in increased opioid SA in animals. It is unclear, however, whether social isolation and food-deprivation are stressors because none of the archived studies report changes in objective indices of the stress response (e.g., plasma catecholamine [Kvetnansky & Mikulaj, 1970] or corticosteroid levels [Kant et al., 1987 Meyerhoff et al., 1988; Selye, 1956]). Further, other studies reported that, in rats, neither isolation (Fagin et al., 1983; Giralt & Armario, 1989; Harrap et al., 1984) nor food-deprivation (De Boer et al., 1989) affect biological parameters of stress such as plasma norepinephrine, ACTH, corticosterone, or blood pressure. In addition, it has been hypothesized that increased drug self-administration under conditions of food-deprivation may be related to variables other than stress, such as the effect of drugs on the hunger drive (Grunberg &

Baum, 1985), or may illustrate a more general phenomenon, "reinforcement interaction," whereby decreased availability of one reinforcer increases responding maintained by another (Carroll & Meisch, 1984).

In sum, there exists suggestive evidence from three animal studies (Dib & Duclaux, 1982; Dib, 1985; Shaham et al., 1992) indicating a positive relationship between stress and opioid SA. However, the lack of additional empirical data from SA studies preclude a firm conclusion concerning a causal link.

Effects of opioid drugs on the stress response in animals

Indirectly, one can make inferences about the relationship between opioids and stress by examining the effects of opioids on physiological and behavioral responses associated with exposure to stress. At the behavioral level, several animals studies have examined the effect of opioids on responses to conditions that can be regarded as stressful to the organism. Leaf and Muller (1965) showed that morphine (1 mg/kg) attenuated the electric shock-induced suppression of drinking behavior in water-deprived rats. Davis (1979) examined the effect of morphine on the potentiated acoustic startle reflex in male rats. This phenomenon refers to the augmentation of the startle reflex by presenting the eliciting acoustic stimuli in the presence of a cue that has been previously paired with a shock. Davis found that morphine attenuated the potentiated startle effect in a dose-dependent manner, and interpreted the results as indicating that morphine may reduce the stress associated with the cue presentation. In contrast, Kelleher and Morse (1964) examined the effect of morphine (1-10 mg/kg) in pigeons trained in a conflict paradigm (i.e., a multiple schedule of reinforcement that includes punished and nonpunished components). These authors reported that, unlike anxiolytic drugs (e.g., benzodiazepines and barbiturates), morphine did not consistently restore responding suppressed by electric shock punishment. Further, Houser (1978), after reviewing the literature on the effects of drugs on behavior controlled by aversive stimuli, concluded that opioid drugs usually do not restore behaviors suppressed by aversive stressful stimuli (usually electric shock). The latter conclusion was further supported by findings reviewed in a more recent article by Barrett

(1987), who indicated that, unlike anxiolytic drugs, opioid agonists do not consistently decrease rate of responding in procedures of shock escape (i.e., the behavioral responding terminates a shock administration). For example, Barrett and Stanley (1983), using monkeys, reported that morphine (0.01-3 mg/kg i.m) increased the rate of shock avoidance responding when operant responding was maintained by a concurrent schedule of shock-postponement and response-produced shock. Therefore, the conflicting evidence of the effect of morphine on behaviors maintained by aversive stimulation does not support predictions based on the stress buffering hypothesis of opioid drugs. In other words, if opioids are being used to cope with aversive environmental events, one would expect that they would affect behaviors maintained by these events.

At the physiological level, although opioid drugs affect numerous bodily systems, this discussion is confined to three physiological systems that play a central role in the stress response, namely the adrenocortical-pituitary hormonal axis, brain norepinephrine pathways, and peripheral catecholaminergic systems (Kant et al., 1987; Kvetnansky & Mikulaj 1970; Redmond & Huang, 1979; Selye, 1956; Tanaka et al., 1983). Contradictory findings exist concerning the effect of morphine on adrenocorticotrophic hormone (ACTH) and corticosterone. Selye (1936) classified morphine (300 mg/kg) as a stressor, capable of activating the corticotropin-ACTH system, because of its effect on the thymus and adrenal cortex of rats. Other authors found that much lower acute dosages of morphine (1-10 mg/kg) and other opioid agonists are capable of increasing corticosterone levels in the plasma (George & Way, 1955; Hayes & Stewart, 1985). In contrast, other studies with rats have reported that chronic morphine administration inhibits the corticosterone and ACTH response to stressors (Buckingham & Cooper, 1984; Munson, 1974). The general conclusion from these studies is that acute morphine administration activates the adrenocortical pathway, whereas chronic morphine administration attenuates or completely abolishes the stress reaction of this pathway. Hence, these data indicate that chronic opioid administration is related to a reduction in the physiological stress response of the adrenocortical-pituitary hormonal axis.

Noradrenergic activation in the locus coeruleus nucleus, located in the dorsal pons, is thought to be one of the major brain mechanisms involved in anxiety and stress (Redmond & Huang, 1979; Tanaka et al., 1983). Tanaka et al. (1983) examined the effect of morphine on noradrenergic activity in several brain areas that receive projections from the locus coeruleus in rats exposed to IM stressor as well as controls. In non-stressed animals, morphine caused a dose-dependent increase in noradrenaline turnover, i.e., increased levels of the noradrenergic metabolite 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) in the hypothalamus, amygdala, thalamus, and hippocampus. In contrast, in the stressed animals, morphine attenuated IM-stressor-induced increases in MHPG levels in those brain areas. Conversely, Abercrombie and Jacobs (1988) found that naloxone, an opioid antagonist, increased noradrenergic activity in the locus coeruleus in cats exposed to IM stressor. Gold and Kleber (1979) and Koob and Bloom (1988) further reviewed evidence indicating that chronic exogenous opioid administration inhibits locus coeruleus firing rate both in vivo and in vitro. Altogether, these results indicate that under stressful conditions, opioids may buffer the effect of stress on central noradrenergic pathways.

The effect of stress on peripheral catecholamine systems is less clear. Endogenous opioid peptides and receptors are widely distributed in the sympathetic nervous system, adrenal medulla, and organs innervated by these systems (e.g., blood vessels and heart) (Cox, 1988). According to Cox (1988), no firm conclusion can be inferred concerning the exact role of opioids in these systems. Specifically, empirical evidence do not support the hypothesis that opioids inhibit sympathetic activation and catecholamine release from the adrenal medulla (Cox, 1988). This conclusion is further supported by the fact that therapeutic dosages of morphine and other opioids have no major effect on blood pressure or cardiac rate or rhythm (Jaffe & Martin, 1990). It should be noted, however, that only future studies can determine whether opioid agonists attenuate the peripheral catecholaminergic response to stress because the effect of these drugs on the latter response has not been examined under conditions of stress.

The animal data presented do not uniformly indicate that opioid drugs

buffer physiological and behavioral responses to stress. There exists evidence supporting the notion that opioid drugs attenuate some physiological responses to stress (i.e., plasma corticosterone release and central catecholamine pathways). In contrast, there is no evidence that opioids systematically attenuate the peripheral catecholaminergic systems associated with the stress response. At the behavioral level, the data indicate that opioid drugs are not always effective in restoring behaviors suppressed by aversive stressful stimuli, or decrease rate of responding when the behavior is maintained by shock termination indicating that opioids may not be effective in coping with some of the behavioral changes associated with exposure to stress.

Stress and opioids: Summary of empirical evidence

The data presented indicate that stress and opioid consumption are positively related. In 12 of the 14 humans studies reviewed, stress was positively related to drug use or abuse. In our laboratory rats increased consumption of morphine and fentanyl solutions during choice days between the drugs and water after exposure to IM stressor. Animal data with other aversive environmental events and other drug types further support the notion that exposure to stress increases drug consumption. Indirect evidence of the effect of opioids on physiological systems (adrenocortical-pituitary hormonal axis, and central noradrenaline systems) and negative mood states (e.g., anxiety and depression) associated with the stress response also support the notion that stress may be related to opioid abuse.

However, the correlational nature of all the human studies presented, the methodological limitations of the human studies related to the measurement of stress, and the lack of additional data from animal research prevent a firm conclusion concerning causality. Further, despite the widely held belief in the stress-buffering/reduction hypothesis, the available data provide tenuous support for this hypothesis. Specifically, opioids do not systematically attenuate peripheral catecholamine functions that are a dominant part of the stress response (Cox, 1988; Jaffe and Martin, 1990). Unlike anxiolytic drugs, opioids do not reliably restore animal behaviors suppressed by aversive stimuli or

suppress behavior maintained by shock escape (Barrett, 1987; Houser, 1978). Further, a large body of evidence (see Jaffe, 1985) implies that the prevalence of mood disorders associated with stress is much higher among opioid addicts, despite opioid use (anxiety, approximately 20%; depression, approximately 50%), than the rates observed in the general population. Mirin et al. (1976) further noted that even under controlled laboratory settings, after the drug SA, heroin addicts manifest a brief period of immediate mood elevation, but after this time period report more depression, dysphoria, and anxiety than a sense of well-being. It could be argued that the observed mood changes are associated with life-style changes resulting from the use of illicit drugs. But, in light of the above mentioned evidence, one may still question the efficacy of opioids in relieving some of the responses to stress or the validity of the stress-buffering hypothesis[8]. Therefore, because the stress-buffering model can, at best, only partially explain the relationship of stress and opioid abuse, it is necessary to consider additional mechanisms that may account for the effect of stress on opioid use and abuse.

Potential mechanisms for the effect of stress on opioid abuse

Several other mechanisms have been suggested to account for the positive relationship between stress on opioid abuse. Shaham and Johanson (in preparation) hypothesized that learning principles derived from classical and operant conditioning paradigms may explain, in part, the positive relationship between stress and opioid abuse. Because this hypothesis is the major focus of this dissertation, it is discussed in details below (see section of stress and learning models of drug abuse).

Another possible mechanism is based on evidence of the effect of stress on opioid receptors (Christie, Chesher & Bird, 1981; Stuckey et al., 1989) and opioid levels (Madden, 1977; Seeger, 1984). Specifically, stress may be associated with increased opioid use because it causes biochemical changes that

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[8] The latter conclusion is not new in the drug abuse literature as can be found in Abraham Wikler's writing: "Even with unlimited supplies of the drug and the privilege of self-injection in amounts and intervals ad libitum, the addict, at least under experimental conditions, is a miserable creature, beset by remorse, guilt and anxiety" (Wikler, 1965 p. 87).

are similar to those associated with the development of opioid tolerance and dependence (Christie, Chesher & Bird, 1981; Madden et al., 1977; Seeger et al., 1984; Stuckey et al., 1989). Therefore, under conditions of stress, a larger dosage of an opioid agonist is needed to achieve a given effect or to maintain normal physiological functioning. Similarly, based on evidence of the effect of stress on the dopaminergic mesolimbic system (Abercrombie et al., 1989; Dunn, 1988; Imperato et al., 1990; Roth et al., 1988) it was suggested that the positive relationship between opioid abuse and stress may occur, in part, because chronic stress may sensitize the mesolimbic dopaminergic system associated with opioid reward. In other words, under conditions of stress, a given dosage of an opioid agonist would achieve a larger rewarding effect (Shaham & Johanson, in preparation).

Based on evidence that stress may decrease urinary pH levels which, in turn, would cause a decrease in nicotine bioavailability[9] (Grunberg & Kozlowski, 1986; Schachter, 1978), it was hypothesized that this phenomenon may also apply to opioids (Cohen et al., 1985). Specifically, increased opioid abuse under stress may result, in part, from reduction of the drug levels in the body (Cohen et al., 1985). Hence, opioid users may increase their drug self-administration because they are experiencing withdrawal as a result of the effect of stress reducing drug bioavailability. Based on a similarity between the physiological and psychological responses to stress and responses to withdrawal from opioids and other drugs of abuse, it has been suggested that opioid addicts and other drug addicts may misattribute the stress response to withdrawal effects and thus increase their drug consumption in order to prevent the "pseudowithdrawal" syndrome (Grunberg & Baum, 1985).

The role of all of these potential mechanisms will not be addressed in the present dissertation for several reasons. First, our review of the literature does not suggest that similarities exist between cellular mechanisms hypothesized

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[9] Evidence that stress decreases nicotine bioavailability is provided by Winders (1990). She demonstrated that exposure to stress causes a significant reduction in plasma nicotine levels and some reduction in brain nicotine levels in rats exposed to chronic nicotine administration by osmotic minipump.

to be associated with opioid tolerance (i.e., down-regulation and desensitization of opioid receptors [Cox, 1990]) and effect of chronic stress on opioid receptors (Shaham & Johanson, in preparation). Specifically, of the 17 studies reviewed in this manuscript no changes in opioid receptor affinities were reported after exposure to stress, and chronic stress was not consistently associated with receptor down-regulation. Concerning the pharmacokinetic hypothesis (i.e., stress causes a decrease in the drug bioavailability), it seems that two factors that are critical for the effect of stress on drug elimination (i.e., increased activity of plasma corticosterone/cortisol (Bousquet, Rupe & Miya, 1965) and alteration in urinary pH (Schachter, 1978)) apparently do not affect the bioavailability opioid drugs such as morphine and heroin. The latter conclusion is based on the inhibitory role of chronic opioids on corticosterone activity (Buckingham & Cooper, 1984), and the fact that morphine plasma levels are not affected by alteration in urinary pH levels (Berkowitz, 1976). Despite the feasibility of the other two mechanisms (i.e., effect of stress on brain reward systems and the misattribution of stress symptoms to withdrawal), examination of the former hypothesis requires techniques in disciplines outside the scope of this dissertation (e.g., microdialysis, receptor binding techniques) and examination of the latter hypothesis requires a special patient population (i.e., opioid addicts) that is also not available.

The present research focused on the examination of the role of conditioning factors in the stress/drug interaction. In the section below, the rationale and previous research pertinent to the hypothesized role of conditioning factors in the relationship between opioids use and abuse and stress are presented.

Stress and learning models of drug abuse

Stress and the conditioned withdrawal-like syndrome

Conditioning factors in drug abuse and relapse were first suggested by Abraham Wikler in the 1940s (Wikler, 1965). Wikler observed that former opioid addicts sometimes show an abrupt onset of withdrawal signs months after their last drug dose. These symptoms often occurred during therapy sessions when the patients discussed their drug use. These observations led Wikler to theorize a

conditioning process that he labeled as "conditioned abstinence." Within the Pavlovian conditioning framework "conditioned abstinence" can be interpreted in the following way: Repeated episodes of withdrawal state (elimination of opioids from the body, the unconditioned stimulus [UCS]), that elicit the withdrawal syndrome (the unconditioned response [UCR]), are paired with environmental stimuli (the conditioning stimuli [CSs]) so that eventually these stimuli provoke a conditioned withdrawal-like syndrome (the conditioned response [CR]) (O'Brien et al., 1986).

Wikler's clinical observations were supported by a series of animal experiments (Goldberg & Schuster, 1970; Hinson et al., 1986; Wikler & Pescor, 1967; Wikler et al., 1971). For example, Wikler and Pescor (1967) paired an environment with the morphine abstinence syndrome that occurred during the night in animals injected with a daily i.p morphine (200 mg/kg) injection for 6 weeks. After termination of the morphine injections, these rats showed higher frequencies of "wet dog" shakes (a withdrawal measure) upon exposure to the environment previously paired with the opioid withdrawal syndrome in comparison to a neutral environment. In another study, Goldberg and Schuster (1970) paired a red light with nalorphine (an opioid antagonist) injection in morphine-dependent monkeys. The nalorphine produced an immediate withdrawal syndrome (e.g., vomiting and excessive salivation). After 10 red light-nalorphine pairings, the morphine was withdrawn for several months. But, in these morphine-free monkeys, the red light continued to elicit the withdrawal syndrome.

Human studies further suggested that drug-related cues are associated with the precipitation of withdrawal symptoms among drug-free and drug-dependent individuals (Donegan et al., 1983). These cues included negative affect (Teasdale, 1973), subjective reports of craving, and increased heart rate and blood pressure (Childress, McLellan & O'Brien, 1986; O'Brien et al., 1975). Altogether, these data indicate that environmental stimuli paired with the withdrawal syndrome or the drug antagonist administration can elicit a withdrawal-like effect even when the opioid addict is detoxified for a long

duration of time or when the opioid drug is continuously available[10].

Stressors may be one of the major environmental events that elicit the withdrawal syndrome after repeated pairing of the stressor with the drug withdrawal. For example, it is assumed that obtaining illegal drugs is a stressful experience. This experience is likely to elicit physiological (e.g., increased plasma levels of catecholamines and cortisol) and psychological (e.g., fear and anxiety) stress responses. Further, it is assumed that the addict frequently obtains the drug while in a state of withdrawal. Under these conditions, exposure to stressors (e.g., meeting a drug dealer) or the stress responses elicited by these stressors (the CSs[11]) and the withdrawal state (the UCS) that elicits the withdrawal syndrome (the UCR) are reliably paired. Therefore, exposure to those stressors or even exposure to the stress responses when elicited by any stressor may in turn elicit the conditioned-like withdrawal syndrome (the CR). Whitehead's (1974) study may lend empirical support to the hypothesis that stress elicits a conditioned-withdrawal syndrome. In this study, methadone maintained patients showed withdrawal symptoms, despite continuing high-dose methadone levels, each time a stressful life event was encountered.

Stress and the conditioned-compensatory response associated with opioid tolerance

An additional learning mechanism that may explain the effect of stress on drug use is that stress may alter conditioned or associative tolerance development to opioid effects. A conditioning model of tolerance was developed by Siegel and colleagues that emphasizes the role of classical conditioning

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[10] In recent years, several researchers challenged the role of physical dependence in the maintenance and relapse to drug abuse. These conclusions were based on evidence that self-administration and conditioned place preference for drugs in animals and drug use in humans can be maintained without the development of physical dependence (Stewart et al., 1984; Wise & Bozarth, 1987). Nevertheless, at least in the case of opioids, physical dependence and conditioned withdrawal may play a role in drug abuse (Jaffe, 1990).

[11] Within the stress arena, stressors can be regarded as UCS and the stress response as UCR. In the present context, however, stressors or stress responses can serve as conditioned stimuli paired with the primary physiological and psychological disturbance, i.e., withdrawal.

(Siegel, 1989). According to this model, the drug, or unconditioned stimulus (UCS), is reliably predicted by a set of environmental stimuli (CSs) related to the drug administration. Following repeated drug administration, the CS associated with the drug elicits a classically conditioned response (CR), often compensatory or opposite, to the drug effect. The conditioned compensatory response is hypothesized to counteract the actual drug effect resulting in a weaker effect to a given drug dosage (i.e., development of tolerance). Siegel's model is compatible with the "opponent process model" of motivation developed by Solomon and Corbit (Solomon & Corbit, 1974). Pertinent to development of drug tolerance, this model argues that each drug administration ("a" process) evokes an opposing reflex reaction ("b" process) that becomes stronger with repeated use and tends to neutralize the effects of the drug, resulting in tolerance development[12].

Numerous studies (see Siegel, 1989) mainly utilizing opioids, provide empirical support for Siegel's model. In general, all of these studies incorporated two groups of rats, both receiving the drug for a sufficient number of times for tolerance to develop during the initial phase of the experiment. The opioid effect (e.g., analgesia) was evaluated in a subsequent tolerance test. For one group, this test was conducted following the same cues that signaled the drug during the tolerance development phase. For the other group, the tolerance test was conducted following different cues than those that signaled the drug during the tolerance development phase (Siegel, 1989). For example, when animals were tested in the same environment in which they were treated with daily morphine, they were tolerant to its analgesic effects. But, when they were tested in a different environment, the degree of tolerance was markedly reduced (Siegel, 1976). Conditioning factors also affected the lethal dose of heroin in rats (Siegel et al., 1982). Direct involvement of conditional compensatory responses in the development of tolerance to the analgesic and hyperthermic

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[12] Opponent process model and Siegel's conditioning model may apply to physical dependence as well. According to Siegel (1989), exposure to the CS alone would elicit a compensatory withdrawal-like response. In the opponent process model, in the absence of the drug, the b process is expressed and drug-opposing effects (withdrawal syndrome) are observed (O'Brien, 1986).

effects of morphine in rats was indicated by the demonstration of hyperalgesic or hypothermic responses after an injection of saline (the CS) (Siegel, 1975, 1978)[13].

As mentioned before, stressors are hypothesized to serve as CS that may be associated with the administration of opioids and other drugs. Stressors may elicit the compensatory CR and contribute to the development of opioid tolerance. Specifically, if the drug administration (the UCS) is reliably associated with stressors or the *stress* responses (the CSs), then following repeated pairing of drug administration and stress, the stress components are hypothesized to elicit the compensatory CR. It should be noted, however, that despite several theoretical developments (see Eikelboom & Stewart, 1982), it is still not possible to reliably predict whether a given environmental stimulus will elicit a conditioned compensatory response to the drug effects or conditioned drug-like effects. But, it is likely that stress will evoke a compensatory response because some of the physiological and psychological responses to stress are opposite to those observed after opioid administration (e.g., sedation vs. arousal, and euphoria vs. negative mood states) (Baum et al., 1987; Grunberg & Baum, 1985; Jaffe & Martin, 1990).

Stress as a discriminative stimulus to drug-seeking behavior

The discussion to this point has been confined to classical conditioning factors in the effect of stress on drug use. Operant conditioning factors also may be involved in the relationship between stress and drug use. Within the operant conditioning paradigm, stressors or stress responses may serve as discriminative stimuli that set the occasion for the drug-seeking behavior. Discriminative stimuli do not elicit responses. Rather, they set the occasion on which responses have consequences (Catania, 1984, p. 127). Therefore, when

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[13] The exact role of conditioned compensatory responses in mediating tolerance and dependence is not firmly established. For example, some of the conditioned responses to stimuli associated with opioid administration (e.g., sight of syringe and saline injection) elicit a conditioned response that is like the unconditioned response (see O'Brien et al., 1986). In addition, in many cases a conditioned tolerance is observed in the absence of a demonstration of a conditioned compensatory response after exposure to a saline injection (see Baker & Tiffany, 1985; Eikelboom & Stewart, 1982; and Goudie & Demellweek, 1986; for reviews).

a given stimulus signals an organism that its behavior may have certain consequences, the organism may come to respond differentially when the stimulus is present than when it is absent. In other words, the behavior of an organism is under the control of a discriminative stimulus or under stimulus control (Catania, 1984).

Evidence suggests that, in animals, environmental events associated with the drug SA may control drug-seeking behavior in the absence of the drug (Bickel & Kelley, 1988; Stewart et al., 1984). For example, Stretch, Gerber and Wood (1971) trained squirrel monkeys to lever press for intravenous infusions of *d*-amphetamine for 2 h a day. During the session, external discriminative stimulus (a green light) was associated with each drug infusion. After an extinction period, lever pressing for saline infusions after intramuscular injections was reinstated only in the presence of the discriminative stimulus previously associated with the drug injection, indicating that the drug-seeking behavior of the animals was under stimulus control. Similar findings were reported by Carroll and Meisch (1979). These investigators trained rats to self-administer oral etonitazene (a potent opioid agonist) solutions. After an extinction phase, higher rates of drug SA were obtained in the presence of a stimulus (a discriminative stimulus) previously associated with the drug SA.

Based on these reports, is hypothesized that stressors may become discriminative stimuli that occasions drug-seeking behavior. For example, as mentioned above, purchasing or dealing with illegal drugs is hypothesized to be a stressful condition. If this process entails repeated exposure to stressors associated with the drug-seeking behavior, then the stressors associated with the drug-seeking behavior may become a discriminative stimulus that occasions that behavior. As the drug-seeking behavior comes, in part, under the discriminative stimulus control of stress, exposure to other stressors (e.g., job stressors, marital problems, etc.) may control the drug-seeking behavior.

In sum, it is hypothesized that learning principles derived from classical and operant conditioning paradigms may explain, in part, the positive relationship between stress and opioid abuse. Within the classical conditioning

paradigm, it is hypothesized that stressors may elicit a conditioned-withdrawal state that is similar to that observed during opioid abstinence. Stressors may further elicit a conditioned compensatory response that is opposite to the drug effect and, by that mechanism, contributes to the development of tolerance to the drug effect. Within the operant conditioning paradigm, it is hypothesized that stressors or the stress response may serve as discriminative stimuli that occasion drug-seeking behavior.

Methodological considerations: Oral opioid self-administration

This section discusses several methodological considerations associated with the oral route of administration, measurement of conditioning effects, and stress. The purpose of this section is to elucidate the conditions necessary to determine whether environmental, behavioral, and pharmacological factors differentially affect the reinforcing properties of orally SA drugs. Two different set-ups are used in the oral paradigm: SA of the drug in the home cage, or drug administration contingent upon a specific response (e.g., lever press) in the operant chamber. These two paradigms assess two different behaviors that may be affected by drug exposure, namely, natural unconditioned drinking behavior, and operant behavior (e.g., lever pressing) conditioned to the drug SA.

The procedure used when the drug is self-administered in the home cage is to initiate drug consumption by making the drug the only solution available. Many studies have reported that opioids (mainly morphine) are readily self-administered via the oral route despite their bitter taste (e.g., Hinson et al., 1986; Nichols et al., 1956; Stolerman & Kumar, 1970). In this paradigm, several factors should be considered before it can be concluded that the drug has served as a reinforcer. These factors include comparison of the drug consumption to water consumption on specified choice days (Nichols et al., 1956; Stolerman & Kumar, 1970), and comparison of drug consumption to consumption of another bitter solution (e.g., quinine) (Stolerman & Kumar, 1970). The utility of the paradigm is strengthened if physical dependence (i.e., demonstration of withdrawal upon opioid antagonist injection or cessation of the drug administration) occurs

(Marks-Kaufman & Lewis, 1984). For example, Stolerman & Kumar (1970) induced morphine or quinine oral SA (7 h/day) by forced consumption of these drugs for 21 days in male rats. After this time period, choice trials were conducted in which both drug and water were available. No differences between morphine and quinine groups were observed on the first choice day (approximately 20% of drug consumption out of the total solution consumption [water+drug solution]). Over time, preference for morphine increased to 55%, whereas preference for quinine decreased to 10-15%. Further, decreased body weight (a withdrawal sign) occurred in the morphine group when the drug solutions were no longer available, suggesting the development of physical dependence. It should be noted, however, that the demonstration of physical dependence is not a necessary criterion for a valid animal model of drug abuse. Specifically, there exist ample demonstrations in the literature for the maintenance and reinstatement of opioid SA in the absence of the development of physical dependence (Bozarth & Wise, 1984; Stewart et al., 1984).

These criteria (i.e., increased preference for the drug during choice days, administration of quinine solutions, and demonstration of physical dependence) should also be applied in the examination of the effect of environmental factors (e.g., exposure to stress) or other factors on oral drug SA. It also is important to demonstrate that the experimental manipulation does not differentially affect taste or thirst mechanisms (i.e., the change in drug consumption must not be an epiphenomenon of other physiological effects). These criteria for opioid reinforcement have not always met in the published literature. For example, although Hadaway et al. (1979) studied oral morphine SA in rats, a close inspection of the study procedure and results indicates that morphine was not established unequivocally as a reinforcer. Specifically, a choice procedure was not utilized, the morphine solution was given in a sweet sucrose solution, and no data were provided on the development of physical dependence.

One limitation of most of the studies that used the oral SA paradigm in a home cage setting is related to the induction of opioid SA by periods of forced

solution consumption. The forced consumption procedure usually results in the development of physical dependence (see Stolerman & Kumar, 1970). Under these conditions it is not possible to delineate the exact mechanism underlying the drug-reinforced behavior. Specifically, it is believed that positive reinforcement mechanisms (i.e., the drug is self-administered because it serves as a positive reinforcer) and negative reinforcement mechanisms (the drug is self-administered in order to avoid or escape the withdrawal syndrome) contribute to opioid SA (Jaffe, 1990). At present, however, the relative contribution of the positive and negative reinforcement components in maintaining drug SA is unclear.

Finally, despite the fact the majority of studies used morphine, this drug has its limitations when administered via the oral route. Morphine is a relatively weak opioid drug in comparison to synthetic opioids (e.g., etonitazene and fentanyl) and for that reason effective dose concentrations in oral solution have a very bitter taste (Jaffe & Martin, 1990). Therefore, relatively limited effective dosage range can be used via this route. Further, oral morphine goes through a significant first-pass metabolism in the liver (Jaffe & Martin, 1990) resulting in lower effective dosage concentration via this route.

Opioids also are self-administered by animals via the oral route using operant techniques (Meisch & Carroll, 1987). In this paradigm, three criteria should be met in order to demonstrate that a drug is serving as a reinforcer: 1) drug presentation must be shown to maintain characteristic patterns of intermittently reinforced behavior; 2) rates of drug maintained behavior must exceed rates of vehicle maintained behavior when the vehicle is presented either sequentially or concurrently with the drug; and 3) pharmacological (e.g., increase/decrease dosages or antagonist administration) or behavioral manipulations (e.g., increasing schedule requirement) should systematically affect rate of response to the drug. Because the initiation phase of drug SA

must include an induction procedure (e.g., schedule-induction[14] or food-induction), it also is important to demonstrate that drug-maintained behavior persists after the removal of these induction procedures (i.e., when food and water are available ad libitum) (Meisch & Carroll, 1987).

Carroll & Meisch (1979) used a food induction procedure in which rats were maintained at 80% of their body weight by a food given for 1 h/day in the operant chamber. After shaping the rats to lever press for water, water was continuously available in the home cage. Next, a dose-response curve (1.25-10 ug/ml) for lever pressing for etonitazene (a potent opioid agonist) was obtained. Subsequently, the food was given after the session and the schedule requirements increased to Fixed Ratio-2, 4, 8 (FR-2, 4, 8). At the highest FR schedule, water was concurrently available with the drug in the operant chambers. In this procedure, higher drug dosages decreased the rate of responding, and during choice days etonitazene was preferred over water, indicating that oral etonitazene can serve as a reinforcer in rats. In subsequent studies Carroll and Meisch (1984) found that oral etonitazene may serve as a reinforcer only under certain conditions (i.e., food-deprivation).

Several other methodological considerations are relevant in the operant paradigm. Because large individual differences in operant responding to the drug are common (Carroll & Meisch, 1984), a within-subject design in which each animal serves as its own control under the different experimental conditions is useful in this paradigm. Second, animals do not always consume the drug solution when the solution is available. As previously reported by Carroll and Meisch (1979), and also observed in our laboratory, sometimes during the drug session animals become involved in non-purposeful stereotyped behavior such as jumping and biting the lever without approaching the drug or water solutions.

Conditioning factors and opioid self-administration

The major purpose of the proposed research is to determine whether

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[14] The most common procedure used to induce drinking is schedule-induced polydipsia, which refers to excessive water or fluid intake that occurs when food-deprived animals receive small pellets of food at the rate of approximately one pellet per minute, Fixed Interval-1 (Falk, 1961).

conditioning factors are involved in the effect of stress on opioid SA. In order to assess the contribution of classical conditioning to the relationship between stress (or any environmental factor) and drug SA, it is important to evaluate the extent to which procedures known to affect classical conditioning (e.g., conditioned inhibition) will modify the effect of stress on drug SA. Operationally, at least three groups of subjects are needed: Control group, not exposed to stress; a conditioned-stress group, in which the stressor reliably predicts or is always paired with the SA period; and, a non-conditioned stress group, in which the animals are exposed to the same stressor duration but the temporal relationship between the stressor and the opioid SA are modified by a procedure that attenuates or abolishes the conditioning effects. Similar experimental designs were used in the past by Siegel and colleagues in the examination of the effect of classical conditioning factors on the development of drug tolerance (see Siegel, 1989).

For example, Siegel et al. (1981) examined the effect of a conditioned inhibition procedure on morphine tolerance. Conditioned inhibition refers to a classical conditioning procedure in which the organism can learn that the CS predicts the absence of UCS. This type of association between CS and absence of UCS is not always detectable because it does not result in overt CR. However, this prior learning results in the retardation of the acquisition of CR when the CS and UCS are subsequently paired (Siegel, 1989). Siegel et al. (1981) demonstrated that the development of analgesic tolerance (as measured by a hot-plate test) was retarded in a group of rats in which the CS was explicitly unpaired (administered 4 h after the drug administration with the morphine injections for 15 days in comparison to animals in which the CS reliably predicted the drug administration). The results of this study provide direct evidence of the involvement of classical conditioning factors in opioid tolerance. Siegel and colleagues (see Siegel, 1989, for a review) further

demonstrated that other procedures (extinction and partial reinforcement[15]) known to attenuate classical conditioning retarded tolerance development of other drug effects (e.g., sedation and thermic effects) and different drug types (e.g., pentobarbital).

One limitation, however, of all of the studies reported by Siegel and his colleagues was that they did not incorporate into their experimental design a "truly random" control procedure (Rescorla, 1967). In this procedure, no learning association of the temporal relationship between the CS and UCS is acquired. Specifically, both the CS and the UCS are presented but there is no contingency between them and no regularity in the temporal occurrence of the events. In other words, the two events are programmed entirely randomly and independently in such a way that any "pairing" of CS and UCS that occurs is merely by chance. This procedure does not have the limitations of several other control procedures for Pavlovian conditioning (e.g., backward conditioning and CS-alone control) that may either introduce nonassociative factors not present in the experimental procedure or may transform the excitatory, experimental CS-UCS contingency into an inhibitory contingency (e.g., a conditioned inhibition procedure (Rescorla, 1967).

Summary of data based on the literature reviewed

Based on the literature reviewed, the following conclusions emerge. Epidemiological reports suggest that stress is positively related to use and abuse of opioid drugs. However, the reasons for this relationship are not clear at present. Further, despite several reports in the animal literature (Dib & Duclaux, 1982; Dib, 1985; Shaham et al., 1992) no firm conclusion concerning a causal link between stress and opioid use can be inferred due to the correlational nature of the human studies and lack of sufficient evidence from the animal literature. It seems that the buffering effect of opioid drugs on responses to stress (i.e., alleviation of the adverse physiological, behavioral,

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[15] Extinction refers to the presentation of the CS without the UCS. Partial reinforcement refers to the presentation of CS-alone interspersed among paired CS-UCS presentations. These two procedures attenuate the CR (Siegel, 1989).

and psychological effects of stress by opioids) provides only a partial explanation for the positive relationship between stress and opioid abuse. The reasons for this conclusion include epidemiological data on prevalence of psychiatric disorders associated with stress, the inability of opioids to affect behaviors controlled by aversive stimuli, and the fact that opioids do not systematically attenuate peripheral catecholamine functions that are a major part of the stress response. Therefore, other physiological and psychological mechanisms may account for the positive relationship between stress and opioid abuse. One of these potential mechanisms, i.e., the involvement of conditioning factors in the relationship between stress and opioid abuse, is examined in this research.

The two main purposes of the dissertation research are to determine whether a causal link exists between stress and increased opioid SA, and to determine whether conditioning factors may mediate the effect of stress on opioid use.

Specific hypotheses

Five major hypotheses were tested in the present experiments:

1) Rats exposed to IM or footshock stressor would increase oral SA of morphine or fentanyl in comparison to control animals not exposed to stress.

2) Higher rates of opioid SA would occur when IM stressor reliably predicts the drug SA period (paired-stress condition) as compared with conditions in which the stressor does not reliably predict the opioid SA period ("conditioned inhibition" and "random pairing" conditions). This hypothesis assesses whether behavioral procedures in which the CS predicts the absence of the UCS (conditioned inhibition) or the CS is randomly paired with UCS (random pairing) would attenuate the effect of stress on increased opioid SA. It was further hypothesized that rate of drug SA in the conditioned inhibition group would be lower than in the random pairing group because in the former group the learned association is that the stressor does not predict drug SA, whereas in the latter group the learned association is that the two events are not related.

3) When the stressor is no longer administered daily, decreased rates of opioid SA would be observed in rats in the stress-drug paired condition, but not in the

other groups. This hypothesis assesses whether the stressor acquires the ability to control drug SA in the paired-stress group.

4) After drug-free and stress-free periods, administration of the stressor to all groups prior to the opioid SA period would result in higher rates of opioid SA in rats in the previous stress-drug paired condition compared with rats in the other groups. This hypothesis assesses whether the previous contingencies between the stressor and the drug SA period would affect "relapse" to drug SA. It was further hypothesized that rate of drug SA would be higher in control animals not exposed to stress in the first phase of the experiment compared with animals exposed to procedures that retard the learning association (conditioned inhibition and random pairing). In addition, in this phase, it was hypothesized that lower rate of drug SA would be observed in the conditioned inhibition group compared with the random pairing group (see hypothesis 2).

5) During drug-free and stress-free periods, exposure to a one-time acute stressor would result in the manifestation of withdrawal symptoms in the paired-stress animals, but not in the other groups. This hypothesis directly assesses whether the stressor previously paired with the drug SA elicits a conditioned withdrawal-like syndrome. Further, it was hypothesized that, during the opioid consumption phase, animals exposed to stress prior to the naloxone injection would manifest a more severe withdrawal syndrome than control animals not exposed to stress.

This study also assessed several corollary hypotheses including:

6) Rats exposed to IM and footshock stressor would have increased plasma corticosterone levels compared with controls. Plasma corticosterone is a biochemical index of the effectiveness of the stress manipulation.

7) Rats self-administering oral opioids would manifest a withdrawal syndrome upon injection of naloxone (an opioid antagonist). The withdrawal assessment is conducted in order to assess whether the animals develop physical dependence to the opioids.

8) Morphine and fentanyl would be detected in plasma of rats that self-administer these drugs. But, based on the literature reviewed (Shaham & Johanson, in

preparation), it was predicted that exposuer to stress would not affect brain or blood levels of those drugs.

EXPERIMENT 1: EFFECT OF stress AND CONDITIONING FACTORS ON MORPHINE AND FENTANYL SELF-ADMINISTRATION

METHODS

Overview

The purpose of this experiment was to examine the effects of IM stressor and conditioning factors on morphine and fentanyl SA in rats. In the first two phases of this experiment, the stressor was either paired (*Paired-stress* [P-S] group), explicitly unpaired (*Conditioned-Inhibition* [C-I] group), or randomly paired (*Random-Pairing* [R-P] group) with the morphine or fentanyl SA. *Control* (C) groups with access to each drug were not exposed to stress. During the last phase of the experiment ("relapse" phase), animals within each of the experimental groups (i.e., P-S, R-P, C-I and C) were divided into two groups (*Control* and *stress*) matched (see Experimental group section below) for their baseline preference for morphine or fentanyl. This experiment examined hypotheses 1 through 8. Opioid solutions were available for 6 h/day (10am-4pm) in the home cage. The main dependent variables were the proportions of opioids consumed of total fluid consumption (water+drug) in the experimental groups (i.e., P-S, C-I, R-P and C groups within each drug) during the choice days (every 5th day) in the initial phase of this experiment (first 50 days, *Phase 1*, including 10 choice days [C1-C10], and 10 forced consumption [FC1-FC10] periods of 4 days each), upon discontinuation of the stressor (days 51-60, *Phase 2*, stress-free phase, including 2 choice days [C11-C12], and 2 FC periods [FC11-FC12]), and after 20 days of a *Drug-Free, stress-Free* period at which time both the stressor and drug were reintroduced, a "relapse phase" (days 81-110, *Phase 3*, including 6 choice days, and 6 FC periods). Additional dependent measures included: opioid and water consumption during choice days; opioid consumption during the FC days in which only the drug solutions were available; the severity of the withdrawal syndrome after naloxone administration in the last week of *Phase 1* of the experiment, after stressor administration during the last week of

the drug-free and stress-free phase, and after naloxone administration in the last week of the "relapse" phase (*Phase 3*); and plasma and whole brain levels of morphine and fentanyl. The effectiveness of the stress manipulation was assessed by assaying plasma corticosterone levels.

Subjects

Sixty-six male Wistar rats (Charles River) were used. Animals were maintained in a temperature (23^0 C) and humidity (50%)-controlled room. Light-dark was a 12-12 hours cycle (lights on 0700-1900), and animals were individually housed. Food (Purina rat chow) pellets were provided ad libitum. Male Wistar rats were used as subjects because in a previous experiment in our laboratory they showed a reliable increase in morphine and fentanyl SA after exposure to stress (Shaham et al., 1992). Increased opioid SA also was observed in Wistar rats after exposure to other aversive environmental events (e.g., food deprivation [Carroll & Meisch, 1984]).

Drugs

Morphine-sulfate powder (Mallinckrodt Inc., St. Louis) in concentrations of either 0.25 or 0.5 mg/ml dissolved in tap water, or fentanyl-HCl (NIDA) in a concentration of 25 ug/ml dissolved in tap water were used. Naloxone-HCl (Dupont Pharmaceutical) in a concentration of 0.4 mg/ml in 0.86% NaCl (saline) solution injected i.p. was used to precipitate the withdrawal syndrome. Naloxone-HCl dosage was 1.0 mg/kg. The morphine and fentanyl concentrations were chosen based on previous studies in our laboratory and other published reports (e.g., Stoleman & Kumar, 1970). The naloxone dosage of 1.0 mg/kg elicited a reliable withdrawal syndrome in our previous studies (Shaham et al., 1992).

Solution consumption schedule

All animals had access to either an opioid solution alone or a choice between this solution and water in cycles of 1 day of choice followed by 4 days of no choice (forced consumption [FC] days). The oral opioids or water were made available for 6 h/day during the forced-consumption and the choice days (between 10am and 4pm). Baseline choices were conducted between 0.5 mg/ml morphine and water or 25 ug/ml fentanyl and water prior to the IM stressor administration.

In the morphine groups, the first choice day between 0.5 mg/ml morphine and water were conducted after 4 days of forced consumption of a diluted solution of 0.25 mg/ml morphine. Three-day baseline water consumption for 24 h/day, and 3-day baseline water consumption for 6 h/day, were determined prior to the start of the experiment. The initial diluted morphine solution was used because some rats do not initiate morphine SA when a higher concentration (a more bitter solution) is initially introduced. Solutions with higher morphine concentrations were not used because they are associated with a general decrease in fluid consumption (S. Holtzman & R. Marks-Kaufman, personal communication, September, 1990). This schedule of opioid consumption was based on previous studies in our laboratories (Shaham et al., 1992).

Experimental groups

In *phase 1* of the experiment, within each drug, four experimental groups (n=8-9) were examined: P-S, C-I, R-P, and C. The number of subjects in each experimental group was determined based on a power analysis provided below (see Statistical and power analysis section). P-S animals were exposed to 15 min of IM stressor just prior to the drug SA period. The C-I animals were exposed to 15 min/day IM stressor administered within a time period of 1-3 hours after the end of the drug SA period during the FC days and during odd choice days (choice days 1, 3, 5, 7 and 9; *Non-Test Choice Days*[16]). The IM stressor administration in this group during even choice days (choice days 2, 4, 6, 8, 10; *Test Choice Days*) were identical to the P-S group (i.e., just prior to the onset of the drug SA period). The R-P groups were exposed to IM stressor randomly administered either before or after the drug SA; that is, the 15 min/day IM stressor was administered at pre-determined 30 min-random time blocks within a window of 0-2 h prior to or 0-3 h after the drug SA. IM stressor administration during the

[16] *Non-Test Choice Days* refers to choice days in which the IM stress administration preceded the drug SA period in the P-S group and followed the drug SA period in the R-P and C-I group. During these choice days only a main effect of stress on opioid SA can be examined. *Test Choice Days* refers to choice days in which the IM stress administration preceded the drug SA period in all of the stressed groups (i.e., R-P, C-I and P-S). During these choice days a main effect of stress and the role of conditioning factors in the effect of stress on drug SA can be examined.

choice days was identical to the C-I group (i.e., after the drug and water SA during *Non-Test Choice Days* [choice days 1, 3, 5, 7, 9], and prior to the drug and water SA period during *Test Choice Days* [choice days 2, 4, 6, 8, 10]). C groups were not exposed to stress, but had access to the opioid solution in exactly the same manner as did the other groups.

During the "relapse" assessment (*Phase 3*), within each drug, animals within each of the previous experimental groups (i.e., P-S, R-P, C-I and C) were divided into two groups (*Control and stress*) matched[17] for their baseline preference for morphine or fentanyl. *Control* groups (n=17 within each drug class; 5 animals from the C group, and 4 animals from each of the R-P, C-I, and P-S groups of *Phase 1*) were not exposed to IM stressor during this phase. *Stress* groups (n=16 within each drug class; 4 animals from each of the C, R-P, C-I, and P-S groups of *Phase 1*) were exposed to 15 min of IM stressor just prior to the drug SA.

The number of choice days were based on previous studies in our laboratory indicating that a clear effect of paired-stress on morphine SA occurs after 9-11 choice days (i.e., 50-59 days). During the *Phase 1* of the experiment, the effect of stress administered prior to the SA period in the C-I and R-P groups was determined only during five choice days in order to prevent the association between the IM stressor and the choice procedure. In other words, because the main dependent variable in this experiment was the proportion of drug solution consumed during choice days, it was necessary that IM stressor in the C-I and R-P groups would not reliably predict the occurrence of the choice days.

Immobilization stressor

IM stressor was administered every day for 15 min. Animals were immobilized by a finger-like immobilization apparatus (Centrap cage, Fisher Scientific) in a nearby room. This stressor was chosen because previous studies conducted in our laboratory (Raygada et al., 1992; Shaham et al., 1992) as well

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[17] Specifically, one day prior to the re-administration of the IM stress and the drugs, the animals' preference for the opioids were assessed during a choice day in which both water and the opioids (morphine or fentanyl) were available for 6 h/day. Animals within each of the experimental groups (i.e., C, R-P, C-I and P-S) were divided into either a *Stress* or *Control* condition. The animals were matched for their drug preference so that the *Stress* and the *Control* groups would be comparable on their baseline preference levels.

as other reports (e.g., Kant et al., 1987) indicated that animals repeatedly exposed to IM stressor, with or without concurrent morphine or fentanyl administration, for durations of 14-59 days, manifest a reliable rise in plasma corticosterone levels (an index of stress) in comparison to controls.

Assessment of physical dependence

Naloxone 1 mg/kg was injected i.p. during the last week of *Phase 1* and the last week of the "relapse" phase (*Phase 3*) to assess the development of opioid dependence. During *Phase 1*, naloxone was injected in the afternoon after the drug SA period. Animals from the P-S, R-P, and C-I groups were exposed to 15 min of IM stressor prior to the naloxone injection. In the "relapse" phase, naloxone was injected in the afternoon after the drug SA period. Animals exposed to IM stressor (the *stress* groups) were exposed to 15 min of IM stressor prior to the naloxone injection. *Control* groups in *Phases 1* and *Phase 3* were not exposed to IM stressor prior to the naloxone injection. During the *Drug-Free, stress-Free* period the withdrawal measures were assessed after all animals were exposed to IM stressor in order to examine whether exposure to IM stressor can elicit the withdrawal syndrome in drug-free animals.

Assessment of the withdrawal syndrome lasted 20 min after the naloxone injection (*Phase 1* and *Phase 3*) or after the exposure to IM stressor (during the *stress-free, Drug-free* period). The assessment was conducted by two independent observers who were not aware of the experimental conditions. The scoring session was conducted in groups of 3-4 animals at a time. The observers were instructed to count the withdrawal symptoms of: 1) wet-dog shakes (rapid and jerky head and shoulders movements), 2) diarrhea (discrete episodes of loose bowel movements), 3) mouthing and teeth chattering (bouts of excessive licking of the mouth or bouts of teeth chattering , 4) ptosis (discrete episodes of drooping of the eyelid), 5) excessive grooming (bouts of excessive licking of body parts), 6) abnormal posture (discrete episodes of body posture or movement that clearly deviate from normal postures or movements), and 7) body weight change 25 min after the naloxone injection. These withdrawal symptoms were based on reports in the literature (e.g., Lewis et al., 1976; Linseman, 1977). This scoring

system resulted in an inter-rater reliability of r=+0.96.

Procedure

The experiment was conducted in four phases: Initial phase (*Phase 1*, days 1-50) including 10 choice days (C1-C10) between the opioids vs. water and 10 FC periods (FC1-FC10) of 4 days each; a stress-free phase (*Phase 2*, days 51-60) including 2 choice (C11-C12) days and 2 FC periods (FC11-FC12) in which the opioids were administered in the absence of stress in all groups; stress-free and drug-free period (days 61-80); and a "relapse" phase (*Phase 3*, days 81-110) including 5 choice days and 5 FC periods in which both IM stressor and the opioids were reintroduced, and an additional 1 choice day and 1 FC period in the absence of stress (see Figure 3). During the "relapse" phase, IM stressor was administered to the *stress* groups just prior to the SA period. The experiment was conducted for 7 days a week.

The provision of morphine or fentanyl solutions or a choice between the opioids and water was conducted as previously described (i.e., cycles of one day of choice between water and drug solution followed by 4 days of FC of the drug). Upon arrival, animals were left in their home cage for 3 days with food and water freely available. This acclimation period was conducted in order to habituate the rats to the novel environment, a factor that is associated with increased drug SA (Piazza et al., 1991a,b). Baseline water consumption was assessed for 6 days prior to the start of the experiment because the opioid drugs are consumed via the oral route. Within each drug class, the groups were matched for their initial drug preference (percent of opioid solution consumed of the total solution [opioid+water] consumed during this day) examined in a choice day conducted prior to the IM stressor administration. That is, animals were divided into the experimental groups such that the groups were comparable on their initial drug preference. Drug solutions were prepared every 10-14 days. Assessment of the development of physical dependence was done as previously described. During *phase 1* and the "relapse" phase, the withdrawal symptoms were assessed in the last week of these phases after the administration of 1.0 mg/kg of naloxone. During the last week of the drug-free and stress-free period, the

withdrawal symptoms were assessed after exposure to 15 min of IM stressor. IM stressor was administered for 15 min per day according to the schedule previously described (see Experimental groups section) The placing of the bottles was counter-balanced daily in the left and right positions of the cages to avoid place preference or aversion to the opioids or water. Both place preference after exposure to opioids (Bozarth & Wise, 1983) and place aversion after exposure to bitter solutions (see Garcia et al., 1974) are factors that may affect drug consumption.

At the end of the experiment animals were decapitated without anaesthesia[18]. Trunk blood was collected in heparinized tubes (50 ul heparin/tube) and centrifuged for 20 min at 1500 x g at 4° C. The brains were removed from the head and were frozen until assayed. All samples were kept at -70° C until assayed. Plasma levels of corticosterone were determined by radioimmunoassay (RIA) (ICN Biomedic). Plasma levels of morphine were determined by RIA (Diagnostic Products Corporation, DPC). For the extraction of brain morphine, brains were homogenized with a 1:1 saline solution in order to extract the drug from the brain tissue, the homogenates were centrifuged (35,500 x g, for 20 min at 4° C) in order to separate the tissue from the supernatants, and the supernatants were used in the RIA procedure (DPC) (a procedure recommended by Dr. Spiller, DPC, and used in our laboratory [Shaham et al., 1992]). Plasma levels of fentanyl were analyzed by a fentanyl RIA for urine (DPC) with the following modifications: The original standards provided by the company were diluted by an equal volume of plasma from a drug-free animal (a procedure recommended by Dr. Whabba, Dept. of Laboratories, Allegheny County, Pittsburgh, and used in our laboratory in the past [Shaham et al., 1992]). For the extraction of brain fentanyl, brains were homogenized with a 2:1, 50% saline / 50% methanol solution in order to extract the drug from the brain tissue, and the homogenates were centrifuged (35,500 x g, for 20 min at 4° Celsius) in order to separate the

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[18] Anaesthesia is known to affect blood and brain levels of a variety of hormones and neurotransmitters including plasma corticosterone (Jean Kant, personal communication, August 1990).

tissue from the supernatant. The supernatants were used in the RIA procedure (DPC) with a similar modification (i.e., the original standards provided by the company diluted by an equal volume of a brain supernatant from a drug-free animal), a procedure recommended by Dr. Whabba, Dept. of Laboratories, Allegheny County, Pittsburgh, and used in our laboratory in the past [Shaham et al., 1992]).

Statistical and power analyses

The following dependent measures were assessed: 1) proportions of morphine or fentanyl solution consumed during choice days, 2) percent of preference change of the drug solution from baseline drug preference during choice days, 3) amount of morphine or fentanyl solution consumed during choice days, 3) amount of water consumed during choice days, 5) amount of drugs consumed during FC days, 6) the withdrawal measures previously described (see Assessment of physical dependence section), 7) plasma levels of the drugs and corticosterone, and brain levels of the drugs. Analyses were conducted separately within each drug class. For variables 1-5, repeated measures analyses of variance were conducted separately within each *phase*. During *phase 1*, analyses of drug preference, drug consumption, and water consumption during choice days were conducted separately for the *Test Choice Days* and *Non-Test Choice Days* because the C-I and R-P groups were exposed to different temporal relationship between the stressor and the drug SA during these days (i.e., prior to the drug SA on the *Test Choice Days* and after the drug SA on the *Non-Test Choice Days*). Simple ANOVAs compared the withdrawal measures in all phases, plasma levels of the drugs and corticosterone, and brain levels of the drugs during *Phase 3*. In case of either main effect of stress condition or stress condition x time interaction, *post hoc* analyses comparing each pair of the experimental groups were done using Duncan multiple range *post hoc* test. Significance level was determined at α=0.05.

Power analysis (Cohen, 1977) using the effect size of the dependent measure of percentages of morphine consumed in our previous studies during the 10th choice day indicated that using 8 animals per group yielded a power of 89% to detect differences between each two experimental morphine groups. Power

calculation based on the effect size of the percentages of fentanyl consumed during the fifth choice day of a previous study (Shaham *et al.*, 1992) indicated that using 8 animals per group yielded a power of 75% to differentiate between each two experimental groups at this time (Cohen, 1977).

RESULTS

Morphine self-administration

Phase 1 - Exposure to immobilization stressor

Before presenting the data analyses, an important methodological point concerning the *Random-Pairing* groups is addressed. Although it was originally intended to use a truly random-pairing control group (Rescorla, 1967) in which the presentation of IM stressor is independent of the drug SA period (i.e., exposure to stress predicts neither the absence *nor* the occurrence of the drug SA period), a close inspection of the experimental procedure indicated that the R-P groups probably were not truly randomly paired. Specifically, in an optimal random-pairing control group, both the stressor administration and the drug SA period should be randomly administered during the 24 h/day period. Due to logistical considerations this schedule was not possible. The drug SA period was conducted at the same time each day, and the stressor administration was conducted within a window of 2 h prior and 3 h after the drug SA period. That is, in about 50% of the drug SA periods IM stressor was presented within 2 hours prior to the drug SA period. It is quite possible, then, that animals in this group learned to associate the morning IM stressor administration with the drug SA period. Therefore, the R-P groups in this experiment may not be viewed as truly random pairing control groups because the morning stressor administration reliably predicted the SA period. Instead, these groups may be viewed as having been exposed to a partial conditioning condition in which a weaker association between the stressor administration and the drug SA period was formed because the conditioned probability for the drug availability after exposure to stress was 50% (i.e., P[drug availability/exposure to stress]=0.5). For the P-S group, in comparison, the conditioned probability for the drug availability after exposure to stress was 100% (i.e., P[drug availability/exposure to stress]=1.0). The

current partial conditioning groups in this experiment were, to a large degree, comparable to the traditional partial reinforcement[19] control group widely used in classical conditioning experiments (Mackintosh, 1983). This procedure is normally adequate to produce a substantial amount of conditioning (Mackintosh, 1983). Therefore, for the rest of this paper the original R-P groups are renamed *Partial-Conditioning* (P-C) groups.

Table 2 presents average dosage levels (mg/kg/day), drug consumption and preference, and water consumption during *Phase 1*. For the FC periods, no significant differences between the groups were observed for morphine consumption. However, as compared to the C group that slightly decreased solution consumption over time, the C-I, P-C, and P-S groups slightly increased their morphine consumption from baseline water consumption levels (see Table 2). This trend is illustrated by the significant effect for stress condition x time [$F(30,290)=2.34$, $p<0.05$].

Test Choice Days

During *Test Choice Days*, the IM stressor was administered prior to the drug SA in the P-S, P-C, and C-I groups. All groups decreased their water consumption over time [$F(5,145)=33.8$, $p<0.05$]. The P-S and the P-C groups increased their drug consumption and preference over time to a greater extent than did the C and C-I groups. Repeated measures ANOVA revealed significant or near significant effects for solution consumption and preference for stress condition [$F(3,29)=4.1$, $p<0.05$, and $F(3,29)=4.2$, $p<0.05$, respectively], for time [$F(5,145)=27.7$, $p<0.05$, and $F(5,145)=40.4$, $p<0.05$, respectively], and for stress condition x time [$F(15,145)=1.93$, $p<0.05$, and $F(15,145)=1.78$, $p<0.06$, respectively] (see also Table 2 for the *post hoc* group differences).

In addition to the analyses of percent of drug preference, an additional analysis examined percent change from baseline drug preference among the treatment groups. Baseline levels were used as covariates in this analysis

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[19] Partial reinforcement procedure refers to a classical conditioning manipulation in which UCS presentations are preceded by a given CS, but CS-alone presentations also interspace between the paired CS-UCS presentations such that the $p(UCS/CS)<1.0$ (Mackintosh, 1983).

because a negative correlation (r=-0.26) was observed between the baseline levels and the average change score during the choice days (i.e., the higher the baseline preference level the lower the change score). Figure 4a presents percent of preference change from baseline levels. As in the case of the raw percent score analysis, the P-S and the P-C groups increased their drug preference to a greater extent than did the C and C-I groups. Significant effects were observed for stress condition [F=(3,28)=5.0, p<0.05], and time [F(4,112)=3.1, p<0.05] (see also Figure 4a for post hoc group differences during individual choice days).

Non-Test Choice Days

During *Non-Test Choice Days*, the IM stressor was administered prior to the drug SA period in the P-S group, and after that period in the C-I and P-C groups. All groups decreased their water consumption over time [F(5,145)=35.3, p<0.05]. The P-S group and to a lesser extent the P-C group increased their drug consumption and preference over time to a greater extent than did the C and C-I groups (see Table 2). Significant effects were observed for stress condition [F(3,29)=5.4, p<0.05, and F(3,29)=4.8, p<0.05, for solution consumption and preference, respectively], and time [F(5,145)=28.4, p<0.05, and F(5,145)=57.4, p<0.05, respectively] (see also Table 2 for post hoc group differences).

In addition to the analyses of the percent of drug preference, an additional analysis compared percent change from baseline drug preference among the treatment groups. Figure 4b presents percent of preference change scores. As in the case of the raw percent score analysis, the P-S and the P-C group increased their drug preference to a greater extent than did the C and C-I groups. Significant effects were observed for stress condition [F=(3,28)=5.0, p<0.05], and time [F(4,112)=3.1, p<0.05] (see also Figure 4b for post hoc group differences during individual choice days).

Withdrawal measures - *Phase I*

Five of the 7 withdrawal measures used are presented below because only one

animal manifested wet-dog shakes, and only 14 of the 33 animals showed ptosis[20] after the naloxone injection. A total withdrawal score was defined as the sum of the number of diarrhea episodes, teeth chattering, excessive grooming, and abnormal posture. The withdrawal measurements are presented in Figure 4c and Table 3. Results indicate that the P-S and the P-C groups manifested a more severe withdrawal score after exposure to IM stressor prior to the naloxone injection as compared to the C-I group exposed to the same treatment and the C group that was not exposed to stress. Significant or near significant differences were observed for the number of teeth chattering and the total withdrawal score [$F(3,29)=2.9$, $p<0.055$, and $F(3,29)=4.4$, $p<0.05$, respectively]. *Post hoc* analyses revealed that the P-S group was significantly different from the C and C-I on both measures, and the P-C group was significantly different from the C and C-I groups for the total withdrawal measure. Though not statistically significant, a similar trend was observed with the other withdrawal measures (see Figure 4c).

Phase 2 - No exposure to immobilization stressor

Table 4 presents average dosage levels (mg/kg/day), drug consumption and preference, and water consumption during *Phase 2*. There were no significant differences for raw consumption data during the two FC periods and the choice days (see Table 4). However, differences between the groups emerged when the groups were compared as to their percent change of drug preference from the last period of *Phase 1*. In this analysis, a change score was computed by subtracting the percent of drug consumption of C11 and C12 from the average percent preference of the last 2 choice days of the *Test Choice Days* (i.e., C8, and C10) where IM stressor was administered prior to the drug SA in the P-S, P-C and C-I groups. The percent of drug preference at the end of *Phase 1* were used as covariates in the statistical analysis because a negative correlation ($r=-0.41$) was observed between the change scores and these scores. Figure 4d presents the change score data. The P-S and P-C groups tend to decrease their drug

[20] No significant differences were observed between the groups for the analysis of the number of ptosis episodes.

preference, whereas the C-I and C groups tend to increase it during *Phase 2* as compared to the last part of *Phase 1*. A near significant effect was observed for stress condition [$F(3,28)=2.82$, $p<0.058$] (see also Figure 4d for the post hoc analyses within individual choice days). An additional analysis examined within-groups preference change over time from the last period of *Phase 1* to *Phase 2*. Significant or near significant increases in drug preference were observed for the C-I and C groups [$F(1,7)=7.1$, $p<0.05$, and $F(1,8)=4.4$, $p=0.07$, respectively]. In contrast, a near significant decrease in drug preference was observed for the P-S group [$F(1,7)=3.7$, $p=0.1$]. Though not statistically significant [$F(1,7)=1.9$, $p=0.2$], a similar trend was observed for the P-C group.

Conditioned withdrawal measurement

During the conditioned withdrawal measurement, animals were stress-free and drug-free for 18 days, and withdrawal symptoms were measured after all animals were exposed to 15 min of IM stressor. Statistical analyses are presented for only two of the seven 7 withdrawal measures used because no animals manifested wet-dog shakes or ptosis and most animals did not manifest the withdrawal symptoms of diarrhea episodes (58%), teeth chattering (70%), and abnormal posture (86%)[21]. The results of the withdrawal measurements are presented in Table 5. Exposure to stress during the *Drug-free, stress-free* period did not elicit the opioid withdrawal syndrome. No significant differences between the groups were observed for either excessive grooming or body weight change.

Phase 3 - Relapse phase

During the "relapse" assessment (*Phase 3*), animals within each of the previous experimental groups (i.e., P-S, P-C, C-I and C) were divided into two groups (*Control* and *stress*) matched for their baseline morphine preference. Two (*stress condition* - *Control* vs. *stress* groups) x 4 (*history of exposure to stress* - C, P-C, C-I and P-S groups) analyses of variance were used for the statistical analyses. The data were analyzed separately for the stress period (5 FC periods and 5 choice days) and for the stress-free period (the 6th FC period and the 6th

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[21] No significant differences between the groups were observed for any of these measures.

choice day) because the *stress* group was not exposed to stress during the 6th choice day and the 6th FC period.

Table 6 presents average dosage levels (mg/kg/day), drug consumption and preference, water consumption, plasma corticosterone levels, and plasma and brain morphine levels in *Phase 3*. IM stressor significantly increased plasma corticosterone levels [$F(1,25)=52.0$, $p<0.05$], but did not affect morphine bioavailability [$F(1,25)=0.0$, ns, and $F(1,25)=0.04$, ns, for plasma and brain morphine levels, respectively].

For FC periods, exposure to IM stressor did not affect fluid consumption. No significant differences were observed between the *Control and stress* conditions (see Table 6). For water consumption during choice days, the subjects decreased their water consumption over time. Significant effects were observed for time [$F(5,125)=47.4$, $p<0.05$, and $F(5,125)=74.9$, $p<0.05$, for the stress and non-stress periods, respectively]. No significant effects of stress condition or history of exposure to stress were observed.

Over time, subjects increased their drug consumption [$F(5,125)=16.6$, $p<0.05$, and $F(5,125)=48.0$, $p<0.05$, for the stress and non-stress periods, respectively] and preference [$F(5,125)=26.9$, $p<0.05$, and $F(5,125)=98.6$, $p<0.05$, for the stress and non-stress periods, respectively]. No significant effects of stress condition or history of exposure to stress were observed.

In addition to the analyses of percent of drug preference, an additional analysis compared the groups as to their percent change from baseline drug preference. Baseline levels were used as covariates in this analysis because a negative correlation ($r=-0.40$) was observed between the baseline levels and the average change score during the choice days. Figure 5a presents percent of preference change from baseline levels. Unlike the analyses of raw percent of drug preference, the data in Figure 5a indicate that during the first 2 choice days the *stress* group increased their drug preference to a greater extent than did the *Control* group. Significant or near significant effects were observed for stress condition [$F=(1,24)=2.9$, $p<0.1$], time [$F(4,96)=4.1$, $p<0.06$], and stress condition x time [$F(4,96)=2.8$, $p<0.05$] (see also Figure 5a for the post hoc

comparisons within individual choice days).

In an additional analysis the groups were compared as to their percent change of drug preference during the choice day after the removal of the IM stressor from the last 2 choice days (choice days 4-5) of exposure to IM stressor. In this analysis, a change score was computed by subtracting the percent of drug preference of the 6th choice day from the average percent preference of the 4th and 5th choice days. The drug preference in the last 2 days of exposure to stress was used as a covariate in the statistical analysis because a negative correlation (r=-0.17) was observed between the change scores and the percent of drug preference of the last 2 choice of exposure to stress. After the removal of the IM stressor, the *stress* group decreased its drug preference by 3.2±2.1 percent, whereas the *Control* group increased its drug preference by 8.5±2.5 percent during this time period [$F(1,24)=10.0$, $p<0.05$]. No significant effects of history of exposure to stress were observed in all of the above mentioned analyses (see Figure 5b).

Withdrawal measures - *Phase 3*

Six of the 7 withdrawal measures used are presented below because no animals manifested wet-dog shakes. The results of the withdrawal measurements are presented in Table 7 and Figure 5c. A total withdrawal score was defined as the sum of the number of diarrhea episodes, teeth chattering, ptosis, excessive grooming and abnormal posture. Exposure to IM stressor exacerbated the morphine withdrawal syndrome. Significant or near significant stress condition effects were observed for the withdrawal symptoms of diarrhea episodes, abnormal posture, body weight change and total withdrawal score [$F(1,25)=8.3$, $p<0.05$; $F(1,25)=3.7$, $p<0.08$, $F(1,25)=8.1$, $p<0.05$, and $F(1,25)=4.2$, $p=0.05$, respectively]. No significant effects of history of exposure to stress were observed on the magnitude of the withdrawal syndrome.

Summary of results - Morphine

The results obtained for morphine SA and withdrawal, with the exclusion of the conditioned withdrawal measurement during the *Drug-free, stress-free* period, support the research hypotheses. Exposure to IM stressor increased opioid SA and

enhanced naloxone-induced morphine withdrawal. Further, the stress-induced enhancement of morphine SA and withdrawal was dependent upon the temporal relationship between the stressor administration and the drug SA period. That is, IM stressor increased opioid SA and withdrawal when it predicted the drug availability (the P-C and P-S groups), but not when it predicted the absence of the drug (the C-I group) as compared to a control condition of no exposure to stress. In addition, the temporal relationship between exposure to stress and the morphine SA period determined consequent drug SA in the absence of the stressor. That is, the P-C and the P-S groups decreased their drug preference, whereas the C-I group increased its drug preference compared with the last period of exposure to stress.

Fentanyl self-administration

Phase 1 - Exposure to IM stressor

Table 8 presents average dosage levels (mg/kg/day), drug consumption and preference, and water consumption during *Phase 1*. All groups increased their drug consumption during the FC period compared with their baseline water consumption levels [$F(10,290)=30.7$, $p<0.05$]. However, the increase in fentanyl consumption from baseline water consumption level was greater for the C-I and P-C groups than for the P-S and C groups (see Table 8). This trend is illustrated in the significant time x stress condition interaction [$F(30,290)=1.64$, $p<0.05$].

Test Choice Days

Water consumption decreased during the choice days in the P-S group, but no change in water consumption was observed for the P-C and C groups, and an increased water consumption was observed in the C-I group (see Table 8). Significant effects were observed for stress condition [$F(3,29)=3.2$, $p<0.05$], and for time [$F(5,145)=2.0$, $p<0.05$] (see also Table 8 for post hoc group differences).

All groups increased their drug consumption over time [$F(5,145)=9.2$, $p<0.05$]. The P-S and P-C groups consumed more fentanyl than did the C and C-I groups, but these differences did not reach statistically significant levels [$F(3,29)=1.62$, $p<0.21$] (see Table 8). Increased drug preference over time was

only observed in the P-S and the P-C groups, but not in the C-I and C groups as compared to their baseline drug preference levels. This trend is illustrated by the significant effect for stress condition x time [$F(15,145)=5.8$, $p<0.05$] (see also Table 8 for post hoc group differences).

In addition to the analyses of percent of drug preference, an additional analysis examined percent change from baseline drug preference. Baseline levels were used as covariates in this analysis because a negative correlation ($r=-0.76$) was observed between the baseline levels and the average change score during the choice days. Figure 6a presents percent of preference change from baseline levels. The data in Figure 6a indicate that, as in the case of the raw percent score analysis, the P-S and the P-C groups increased their drug preference and the C and C-I groups did not. The statistical analysis revealed significant or near significant effects for stress condition [$F=(3,28)=3.0$, $p<0.05$], for time [$F(4,112)=3.8$, $p<0.05$], and for stress condition x time [$F(12,112)=1.6$, $p<0.1$] (see also Figure 6a for post hoc group differences within individual choice days).

Non-Test Choice Days

Water consumption decreased in the P-S group, no change in water consumption was observed for the P-C and C groups, and <u>increased</u> water consumption was observed in the C-I group. The statistical analysis revealed significant or near significant effects for stress condition [$F(3,29)=2.5$, $p<0.09$], and for stress condition x time [$F(15,145)=2.4$, $p<0.05$] (see also Table 8 for post hoc group differences).

All of the stress groups increased their drug consumption as compared to the C groups. However, only the P-S and the P-C groups, but not the C-I group, significantly increased their drug preference as compared to the C group (see Table 8). The statistical analyses revealed significant or marginally significant effects for time [$F(5,145)=9.2$, $p<0.05$, and $F(5,145)=6.1$, $p<0.05$, for drug consumption and preference, respectively], and for time x stress condition [$F(15,145)=1.83$, $p<0.05$, and $F(15,145)=1.73$, $p<0.051$, respectively] (see also Table 8 for post hoc group differences).

In addition to the analyses of percent of drug preference, an additional analysis examined percent change from baseline drug preference. Figure 6b presents percent of preference change from baseline levels. As in the case of the raw percent score analysis, only the P-S and the P-C groups increased their drug preference, but not the C and C-I groups. Significant or marginally significant effects were observed for stress condition [$F=(3,28)=3.4$, $p<0.05$], and for stress condition x time [$F(12,112)=1.72$, $p<0.08$]. (see also Figure 6b for post hoc group differences within individual choice days).

Withdrawal measures - *Phase 1*

As in the case of morphine withdrawal during *Phase 1*, 5 of the 7 withdrawal measures used are presented below because no animals manifested wet-dog shakes, and only 4 out of the 33 animals showed ptosis after the naloxone injection. The results of the withdrawal measurements are presented in Figure 6c and Table 9. Unlike the morphine withdrawal data, no significant differences were observed for the withdrawal measures. However, it should be noted that a similar trend observed in the morphine withdrawal data also was observed with the fentanyl groups (see Figure 6c). That is, the results suggest that the fentanyl groups of P-S and the P-C groups manifested a more severe withdrawal score after exposure to IM stressor prior to the naloxone injection as compared to the C-I group exposed to the same treatment and the C group not exposed to stress prior to the naloxone injection.

Phase 2 - No exposure to IM stressor

Table 10 presents average dosage levels (mg/kg/day), drug consumption and preference, and water consumption during *Phase 2*. For the FC period, the C-I group, and to a lesser extent the P-C group consumed higher drug solutions during the FC periods as compared to the C and P-S groups. Further, the C, P-C and P-S groups increased their drug consumption in FC12 as compared to FC11. These trends are illustrated by the significant effects for time [$F(1,29)=12.2$, $p<0.05$], and for stress condition x time [$F(3,29)=2.9$, $p=0.05$] (see also Table 10 for the post hoc group differences).

During the choice days, no significant differences were observed for water

consumption. For drug consumption and proportion, the data suggest that after the removal of the stressor, the C-I group manifested higher SA rates as compared to the C and P-S. The C-I group was not statistically different from the P-C group. The statistical analysis revealed near significant effects for stress condition [$F(3,29)=2.3$, $p=0.1$, and $F(3,29)=2.0$, $p=0.14$, respectively] (see also Table 10 for the post hoc group differences).

Additional analysis examined the percent change of drug preference from the last period of *Phase 1*. Again, in this analysis a change score was computed by subtracting the percent of drug consumption of C11 and C12 from the average percent preference of the last 2 choice days of the ***Test Choice Days***. Also, the latter were used as covariates in the statistical analysis because a negative correlation ($r=-0.58$) was observed between the change scores and the percent of drug preference at the end of *Phase 1*. Figure 6d presents the change score data. The data indicate that the P-S group and to a lesser extent the P-C group tend to decrease their drug preference, whereas the C-I group tend to increase it during *Phase 2* as compared to the last part of *Phase 1*. No systematic trend was observed for change in drug preference in the C group. Statistical analysis of the percent of preference change revealed a significant effect for stress condition [$F(3,28)=4.9$, $p<0.05$] (see also Figure 6d for post hoc group differences within individual choice days). An additional analysis examined within-groups preference change over time from the last period of *Phase 1* to *Phase 2*. A near significant increase in drug preference was observed for the C-I group [$F(1,7)=3.0$, $p=0.13$]. In contrast, significant or near significant decreases in drug preference were observed for P-S and P-C groups [$F(1,7)=13.2$, $p<0.05$, and $F(1,7)=4.6$, $p=0.07$, respectively]. No significant time effect [$F(1,7)=0.8$, ns], was observed for the C group.

Conditioned withdrawal measurement

During the conditioned withdrawal measurement, animals were stress-free and drug-free for 18 days, and withdrawal symptoms were measured after all animals were exposed to 15 min of IM stressor. Statistical analyses are presented for only two of the seven 7 withdrawal measures used because no animals manifested

wet-dog shakes or ptosis and most animals did not manifest the withdrawal symptoms of diarrhea episodes (64%), teeth chattering (79%) and abnormal posture (97%)[22]. The results of the withdrawal measurements are presented in Table 11. No group differences were observed for the analyses of the stress-induced withdrawal symptoms of excessive grooming and body weight change indicating that stress failed to elicit a conditioned withdrawal syndrome in the fentanyl animals.

Phase 3 - Relapse phase

During the "relapse" assessment (*Phase 3*), animals within each of the previous experimental groups (i.e., P-S, R-P, C-I, and C) were divided into two groups (*Control* and *stress*) matched for their baseline fentanyl preference. A 2 (*stress condition* - *Control* vs. *stress* groups) x 4 (*history of exposure to stress* - C, P-C, C-I, and P-S groups) analyses of variance were used for the statistical analyses. The data were analyzed separately for the stress period (5 FC periods and 5 choice days), and for the stress-free period (the last FC period and the last choice day).

Table 12 presents average dosage levels (mg/kg/day), drug consumption and preference, water consumption, plasma corticosterone levels, and plasma and brain fentanyl levels in *Phase 3*. IM stressor caused a significant increase in plasma corticosterone levels [$F(1,25)=22.6$, $p<0.05$] indicating that the stress manipulation was effective. The stress manipulation, however, did not affect the drug bioavailability. No significant differences were observed between the *Control* and *stress* groups for plasma and brain fentanyl levels [$F(1,25)=0.7$, ns, and $F(1,25)=0.9$, ns, respectively].

For FC periods, a comparable increase in fentanyl consumption from baseline water consumption level was observed in the *Control* and *stress* groups over time [$F(5,125)=18.0$, $p<0.05$, and $F(1,25)=24.7$, for the stress and non-stress periods, respectively]. It should be further noted that large individual differences in fentanyl consumption (range: 29-80 ml/day) were observed. That is, some animals

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[22] No significant differences between the groups were observed for any of these measures.

increased their solution consumption during exposure to fentanyl by more than 200% as compared to their baseline 6 h water consumption. No significant effects of history of exposure to stress were observed.

During the first 5 choice days (the stress period), the *stress* group decreased its water consumption to a greater extent then did the *Control* group (see Table 12). The statistical analysis revealed significant effects for stress condition [$F(1,25)=6.5$, $p<0.05$], time [$F(5,125)=15.8$, $p<0.05$], and stress condition x time [$F(5,125)=4.8$, $p<0.05$]. In the last choice day (after the removal of the IM stressor), all animals decreased their water consumption as compared to their baseline choice water consumption [$F(1,25)=30.7$, $p<0.05$]. No significant effects of history of exposure to stress were observed.

For drug consumption during the first 5 choice days, a slight overall increase over time was observed [$F(5,125)=2.4$, $p<0.05$]. Though not statistically significant due to large individual variation in drug consumption during the choice days (*Control* group range: 3-87 ml/day, and *stress* group range: 7-74 ml/day), the data further indicate that during the last 2 choice days of the stress period, the *stress* group (30 ml/day) tended to increase its drug consumption as compared to the *Control* group (23 ml/day). No significant differences were observed for drug consumption after the removal of the IM stressor. Further, no significant effects of history of exposure to stress were observed.

For drug proportion during the first 5 choice days, the *stress* group increased their drug preference over time, whereas no change in drug preference over time was observed in the *Control* group (see Table 12). Significant effects were observed for stress condition [$F(1,25)=5.4$, $p<0.05$], for time [$F(5,125)=2.7$, $p<0.05$], and for stress condition x time [$F(5,125)=3.8$]. No significant differences were observed for drug proportion after the removal of the IM stressor. Further, no significant effects of history of exposure to stress were observed. In addition to the analyses of percent of drug preference, an additional analysis examined percent change from baseline drug preference. Again, baseline levels were used as covariates in this analysis because a

negative correlation (r=-0.73) was observed between the baseline levels and the average change score during the choice days. Figure 7a presents percent of preference change from baseline levels. As in the case of the raw percent score analysis, the *stress* group increased their drug preference over time, whereas no change in drug preference over time was observed in the *Control* group. Significant or near significant effects were observed for stress condition [F=(1,24)=9.8, $p<0.05$], time [F(4,96)=2.4, $p<0.06$], and for stress condition x time [F(4,96)=2.8, $p<0.05$]. No significant differences were observed for percent of preference change after the removal of the IM stressor. However, differences between the *Control* and *stress* groups emerged when the groups were compared as to their percent change of drug preference after the removal of the stressor from the last 2 choice days (choice days 4-5) of exposure to IM stressor. In this analysis, a change score was computed by subtracting the percent of drug consumption of the 6th choice day from the average percent preference of the 4th and 5th choice days. After the removal of the IM stressor, the *stress* group decreased its drug preference by 15.4±3.1 percent, whereas the *Control* group increased its drug preference by 4.5±3.1 percent during this time period. A significant effect was observed for stress condition [F(1,24)=6.0, $p<0.05$]. No significant effects of history of exposure to stress were observed in all of the above mentioned analyses (see Figure 7b).

Withdrawal measures - *Phase 3*

The results of the withdrawal measurements are presented in Table 13 and Figure 7c. A total withdrawal score was defined as the sum of the number of wet-dog shakes, diarrhea episodes, teeth chattering, ptosis, excessive grooming and abnormal posture. Exposure to IM stressor enhanced the fentanyl withdrawal syndrome. Significant stress condition effects were observed for the withdrawal symptoms of wet-dog shakes, teeth chattering, body weight change and total withdrawal score [F(1,25)=7.7, $p<0.05$; F(1,25)=4.7, $p<0.05$, F(1,25)=9.4, $p<0.05$, and F(1,25)=8.3, $p<0.05$, respectively]. No significant effects of history of exposure to stress were observed on the magnitude of the withdrawal syndrome.

Summary of results - Fentanyl

The results obtained for fentanyl SA and withdrawal, with the exclusion of the conditioned withdrawal measurement during the *Drug-free, stress-free* period, and the withdrawal measures during *Phase 1*, support the research hypotheses. Exposure to IM stressor increased fentanyl SA and enhanceed naloxone-induced fentanyl withdrawal during *Phase 3*. Further, the stress-induced enhancement of fentanyl SA was dependent upon the temporal relationship between the stressor administration and the drug SA period. That is, IM stressor increased fentanyl SA when it predicted the drug availability (the P-C and P-S groups), but not when it predicted the absence of the drug (the C-I group) compared with a control condition of no exposure to stress. In addition, the temporal relationship between exposure to stress and the fentanyl SA period determined consequent drug SA in the absence of stress. That is, the P-S group, and to a lesser degree the P-C groups, decreased their drug preference, whereas the C-I group increased its drug preference compared with the last period of exposure to stress.

DISCUSSION

This experiment examined two main hypotheses. First, it was hypothesized that exposure to stress would increase opioid SA and opioid withdrawal in rats. Second, it was hypothesized that conditioning factors mediate, in part, the effects of stress on opioid SA and withdrawal. In general, the results obtained support these hypotheses. During *Phase 1* rats exposed to 15 min/day of IM stressor that was either paired or partially paired (the P-S and P-C groups) with the SA period increased their preference for morphine or fentanyl solutions compared with non-stressed controls. In addition, during the relapse phase, a large increase in drug preference during the choice days was observed in the fentanyl animals exposed to stress as compared to controls. A similar trend was observed for the morphine animals exposed to IM stressor, but the magnitude and the consistency of this stress effect were weaker as compared to the fentanyl groups. These results replicate previous findings from our laboratory (Shaham et al., 1992) and other published reports (Dib and Duclaux, 1982; Dib 1985) suggesting that rats exposed to stress (IM or electric footshock) increase their

opioid SA.

In addition, during *Test Choice Days* of *Phase 1*, when all animals in the stress conditions (i.e., P-S, P-C and C-I) were exposed to stress prior to the drug SA period, an increase in drug preference was only observed in the P-C and P-S groups, but not in the C-I groups as compared to the C groups. These results suggest that the temporal relationship between exposure to stress and the drug availability may mediate, in part, the effect of stress on opioid SA.

Exposure to IM stressor also exacerbated the precipitated opioid withdrawal syndrome induced by a naloxone challenge. During *Phase 1 and 3* of this experiment, animals exposed to stress prior to the naloxone challenge manifested a more severe withdrawal syndrome compared with control animals not exposed to stress prior to the naloxone administration. These results replicate previous reports (Whitehead, 1974; Williams et al. 1984), suggesting that stress may enhance the opioid withdrawal syndrome. The results obtained for the morphine groups further suggest that conditioning factors may also be involved in the effect of stress on opioid withdrawal. Specifically, during *Phase 1* the P-S and P-C groups manifested a more severe withdrawal syndrome than did the C-I and C groups. Therefore, it seems that the learned association between exposure to stress and the drug availability may play a role in the effect of stress on opioid withdrawal.

During the *stress-free, Drug-free* period, all animals were exposed to IM stressor in order to examine whether stress previously paired with the drug SA period can elicit the opioid withdrawal syndrome. Based on previous reports indicating that environmental events previously paired with opioid withdrawal or opioid SA elicit a conditioned-withdrawal syndrome during drug-free periods (O'Brien et al., 1986; Wikler & Pescor, 1967), it was expected that a conditioned withdrawal syndrome would be observed in the P-S and P-C groups but not in the C-I and C groups. The results obtained do not support this research hypothesis. That is, the treatment groups previously exposed to different conditions of temporal relationship between the IM stressor and the drug SA period did not differ in the magnitude of their withdrawal symptoms that was weak in all groups.

The fact that the temporal relationship between exposure to stress and the drug SA period mediate, at least in part, the effect of stress on precipitated withdrawal (the withdrawal syndrome observed after naloxone administration) during drug exposure, but did not affect the manifestation of the conditioned withdrawal syndrome after exposure to stress during a drug-free period appears to be inconsistent. However, several issues must be addressed before it can be concluded that exposure to stress which predicts the drug SA period is not likely to elicit the withdrawal syndrome during a drug-free period.

First, the experimental procedure used in this experiment was not optimal to detect a conditioned-withdrawal syndrome. That is, prior to the *stress-free, Drug-free* period the animals were not exposed to stress but instead continued to self-administer the opioid. Specifically, during *Phase 2* that preceded the conditioned withdrawal measurements, animals consumed the opioids for 10 days in the absence of exposure to stress. In other words, exposure to stress no longer predicted drug availability in the P-C and P-S groups. Therefore, *Phase 2* can be viewed as an "extinction" phase for the relationship between exposure to stress and the drug availability. This putative extinction of the learned association between exposure to stress and the drug availability may be related to the lack of effect of stress on opioid withdrawal during the *Drug-free, stress-free* period.

In addition, the discrepant results between the precipitated withdrawal and the conditioned withdrawal may be related to a more general phenomenon, namely that it is much more likely to find subtle physiological or behavioral changes in drug studies if a challenge test is utilized (Poulos & Cappell, 1991). For example, many studies failed to demonstrate a conditioned compensatory response (Siegel, 1989) to a given drug effect if a placebo is administered instead of the drug in an environment in which the drug is originally injected (see Baker & Tiffany, 1985; Goudie & Demellweek, 1986). This lack of a conditioned compensatory effect after a placebo injection was observed by Hinson et al. (1982) examining the conditioned compensatory response (hyperexcitability) to the sedative effects of pentobarbital. However, using a challenge test with cocaine

(a drug that causes hyperexcitability) these authors reported that the animals showed increased locomotor activity in an environment in which pentobarbital was previously administered as compared to an environment in which a placebo was injected. Hinson et al. (1982) interpreted these findings to indicate that the conditioned compensatory effect observed after repeated exposure to pentobarbital in the drug injection environment summated with the unconditioned excitatory effect of cocaine resulting in higher levels of hyperexcitability.

Taken together, Hinson et al. (1982) as well as other reports (see Poulos & Cappell, 1991) suggest that the use of a challenge test is a more sensitive procedure to detect behavioral and physiological effects of drugs. By analogy, in the present experiment the naloxone precipitated withdrawal can be viewed as a challenge test for the effect of stress and conditioning factors on drug withdrawal. In contrast, the conditioned withdrawal measurement during the *stress-free, Drug-free* did not involve the use of a challenge test, and thus may be less sensitive in detecting the putative effects of stress and conditioning on opioid withdrawal. Therefore, it is suggested that future studies examining the stress-induced conditioned withdrawal syndrome should include a challenge test (e.g., antagonist administration after exposure to stress) during the *stress-free, Drug-free* period.

In any study of the effect of stress on bitter oral drug SA under limited fluid access, it is important to rule out alternative hypotheses that may account to the stress-induced increase in drug SA. Two possible physiological mechanisms that are operating in the oral SA paradigm that may be affected by stress are taste and thirst. That is, if exposure to stress results in a decreased sensitivity to a bitter taste or results in increased thirst, the increased opioid SA under conditions of stress cannot be attributed to changes in the reinforcement efficacy of the oral opioid under conditions of stress. Concerning the effect of stress on sensitivity to a bitter taste, it was previously demonstrated that IM stressor *per se* did not affect the preference of a bitter quinine (0.3 mg/ml) solution as compared to a control condition of no exposure to stress (Shaham et al., 1992). This finding indicates that stress-induced

morphine SA is not due to the fact that the animals in the stress condition are less sensitive to the bitter taste solution.

The effect of stress on thirst in this paradigm can be determined by examination of the solution consumption data during the FC days. Significant differences between the fentanyl groups were observed during these days. Specifically, the C-I and P-C groups increased their solution consumption to a greater extent than did the C and P-S groups on FC days. However, these differences are not likely to be related to the stress-induced increase in drug preference during the choice days because the P-S group increased its drug preference to a much greater extent than did the C and C-I groups during the choice days of *Phase 1* (see Table 8 and Figures 6a and 6b). In addition, there was no difference between the C and P-S groups during the FC days (see Table 8). Also, no overall consumption differences during the FC days were observed between the *Control* and *stress* groups during the relapse phase (see Tables 6 and 12). Moreover, in the fentanyl groups the largest increase in fentanyl FC was observed in the C-I group. However, during *Phase 1*, as compared to the P-S and P-C group, the C-I group only slightly increased its drug preference over time (see Figures 6a and 6b). Taken together, these observations suggest that the stress-induced changes in solution consumption during the FC days in the fentanyl groups of P-C and C-I are not likely to be related to the effect of stress on thirst.

An additional effect on solution consumption was observed in the fentanyl groups during the FC days. Over time, there was an overall increase in fentanyl consumption compared with baseline water consumption and compared with water consumption during the *stress-free, Drug-free* period (see Tables 8, 10 and 12). The reasons for the increased solution consumption observed in the fentanyl groups are less clear. It may be that the fentanyl-induced solution consumption was related to its effect on water balance. Specifically, although urine output was not measured in this experiment it may be that fentanyl caused increased diuresis (increased urination) by inhibiting ADH (antidiuretic hormone [vasopressin], a posterior pituitary hormone that stimulates water reabsorption) release. Previous reports suggest that the effect of *mu* opioid agonists on ADH

release and urine output is time dependent. Specifically, acute administration may cause antidiuresis (Tsushima et al., 1990) or no change in urine output (Leander, 1983), whereas chronic drug administration causes diuresis (Holmes et al., 1958). However, because neither urine output nor ADH levels were measured in this experiment, the effect of chronic oral fentanyl consumption on water balance can only be determined by future research. It should be noted that no change in fluid consumption was observed for the morphine animals despite chronic exposure to the drug. One possibility for the lack of effect of morphine on fluid consumption is that morphine at the concentrations used in this study is a bitter solution, so taste may limit the amount of daily solution consumption in the morphine animals.

An unexpected finding in this experiment was the increase in drug preference observed in the C-I groups after the removal of the stressor during *Phase 2*. Behavioral history studies on the effect of previous exposure to aversive stimuli and controllability over these events on subsequent effects of drugs of abuse (Barrett, 1987; Nader et al., 1992) may be related to this paradoxical aftereffect of exposure to stress on drug SA observed in the C-I groups. For example, in monkeys the rate-decreasing effect of *d*-amphetamine on punished responding can be reversed by prior training on a shock avoidance schedule (Barrett, 1977). This effect was not produced by exposure to the shock *per se*, but was dependent upon the contingencies of the avoidance schedule. That is, only a schedule of a contingent shock avoidance, but not exposure to a non-contingent shock was associated with a subsequent increased rate of responding in the punished procedure (Barrett & Witkin, 1986). Many methodological differences exist between the present experiment and Barrett's studies, however, they both may demonstrate that exposure to stress *per se* is <u>not</u> the critical factor in the stress-drug interaction. In contrast, the relationship between the organism's behavior and the exposure to stress may be the critical factor that determines whether stress will alter drug effects and drug use. Specifically, factors such as controllability over the aversive event, and its temporal relationship with the drug availability may determine the way stress will alter

drug effects and drug use. In light of Barrett's findings of the role of controllability over aversive events in the manifestation of drug effects, an important extension of the present experiment would be to examine this factor (i.e., controllability) in the effect of stress on opioid SA and withdrawal.

As mentioned before (see Introduction section) Siegel's (1989) or Wikler's (1965) conditioning models may be pertinent to the effect of stress on increased drug SA. Therefore, it is important to determine to what extent the results obtained in the present experiment support these conditioning models of drug tolerance and dependence. However, before addressing the applicability of the present experiment results to Siegel's and Wikler's models, it should be noted that in recent years several issues were raised concerning the validity of these models (see Baker & Tiffany, 1985; Goudie & Demmelweek, 1986; O'Brien et al., 1990; Stewart & Eikelboom, 1987). One issue relevant to the findings of this experiment is the lack of experimental evidence that conditioned tolerance or conditioned withdrawal responses are related to increased drug SA or drug relapse (Hinson et al., 1986; Sobrero & Bouton,1989; Thompson & Ostlund, 1965; Wikler & Pescor, 1967). For example, in an examination of Wikler's model of drug relapse, Wikler and Pescor (1967), while demonstrating a conditioned withdrawal in an environment previously paired with morphine withdrawal, failed to demonstrate that this conditioned withdrawal was related to increased oral etonitazene SA in rats. Similarly, using rats, Hinson et al. (1986) demonstrated conditioned analgesic tolerance and increased morphine FC in an environment previously paired with morphine injection or SA, but failed to show an increase in oral morphine preference regardless of the environment.

The present experiment, unlike the previously mentioned studies, found an increased drug SA upon exposure to an environmental stimulus (IM stressor) that was dependent on whether the stimulus predicted, at least to a certain degree, the occurrence (e.g., the P-S and P-C groups) of the drug SA period in accordance with predictions made by conditioning models of drug abuse. The reasons for the discrepant results between this experiment and other published reports are not entirely clear. From a methodological point of view, the incorporation of many

more choice days in this experiment may explain the discrepancy between the present study and the Hinson et al. (1986) and Sobrero and Bouton (1989) studies that used only 1 or 2 choice days during their morphine preference assessment period, respectively. This distinction (i.e., number of choice days), however, cannot explain the discrepant results with Wikler and Pescor (1967) in which a much longer (several months) duration of assessment was used. At a more speculative level, it may be that the context-specific increase in opioid SA observed in this experiment is related to the type of the environmental event used. That is, the fact that the previously mentioned studies did not find environmental-specific effects on drug SA may be related to the fact that they paired a "neutral" environment (e.g., illumination conditions, specific room), whereas the present experiment used a more meaningful aversive environmental event (IM stressor). Indirect support for the above mentioned speculation can be found in Childress et al. (1986). These authors reported that in the course of their study on conditioned withdrawal symptoms in humans, they incidently observed that non-responder opioid abusers (subjects that did not show conditioned withdrawal during most of the experimental sessions) exhibited conditioned withdrawal to drug stimuli *only if* they were tested in a certain negative emotional states associated with stress (e.g., anxiety, depression) (Childress et al., 1986, p. 422).

Despite the fact that a context-specific increase in drug SA was observed in the present experiment (i.e., the effect of stress on opioid SA was only observed when the stressor was paired with the drug SA period), several issues must be resolved before it can be concluded that the results of this experiment clearly support Siegel's or Wikler's models. First, no evidence was observed for the manifestation of conditioned withdrawal upon exposure to stress and a conditioned compensatory response was not examined. The demonstration of these two phenomena and their relationship to increased drug SA are crucial to the application of Wikler's and Siegel's models, respectively, to drug SA. Second, it may be that operant conditioning factors (i.e., discriminative stimulus control) also are operating in the effect of stress on oral opioid SA (see

Introduction section). Further, classical conditioning processes are thought to affect discriminative stimulus control in the operant conditioning paradigm (Mackintosh, 1983; Rescorla, 1969). Hence, it is likely that to the extent the exposure to stress is a discriminative stimulus for opioid SA, a conditioned inhibition procedure may also attenuate its effect on operant drug SA. Taken together, it is not clear, at present, whether classical conditioning processes, operant conditioning processes or both processes are responsible for the results obtained in this experiment.

Another important issue raised by the results of this experiment is the relative contribution of conditioned factors and unconditioned factors in the effect of stress on opioid SA. Unconditioned factors refer to effects of stress on other bodily systems that may affect drug SA. Specifically, exposure to stress elicits a variety of unconditioned responses at the physiological, behavioral, and psychological levels (Amit & Galina, 1986; Axelrod & Reisine, 1984; Baum et al., 1987; Kalivas & Stewart, 1991; Kant et al., 1987; Lazarus, 1966; Mason, 1975; Selye, 1956; Willner, 1984; Zelman et al., 1985). Involvement of unconditioned factors in the effect of stress on drug SA was reported by Piazza et al. (1991a,b). These authors reported that increased *d*-amphetamine i.v. SA was observed even when the drug SA period was conducted one week after the exposure to stress (tail pinch or social competition) or after exposure to prenatal stress. Undoubtedly, conditioning factors in the effect of stress on drug SA are not likely to be involved in the above mentioned studies.

It is further possible that unconditioned factors also were operating in the present experiment. This assertion is based on the fact that several predictions based on conditioning theories concerning the effect of conditioned inhibition procedure were not met in this experiment. Specifically, a typical finding in the use of this procedure is that if a conditioned inhibitor stimulus is presented along with the UCS or a paired CS it results in a subsequent inhibition of the magnitude of the UCR or the CR, respectively (Pavlov, 1927/1960; Rescorla, 1969). However, there were no changes in drug preference in the C-I groups during the *Test Choice Days* (in which a conditioned inhibition

effect is expected because exposure to stress precedes the drug SA period) vs. the *Non-Test Choice Days* (where no conditioned inhibition effect is expected because the stressor administration followed the drug SA period) [$F(1,6)=0.05$, ns., and $F(1,6)=1.5$, ns., for the morphine and fentanyl C-I groups, respectively]. Further, during *Test Choice Days*, the C-I groups did not differ from the C groups (see Figures 4a and 6a). Hence, this set of data do not conform to a prediction of a conditioning model arguing that if only conditioning factors are involved in the effect of stress on drug SA, a decrease in drug preference would be observed in the C-I groups when exposure to stress precedes the drug SA period. The second commonly observed effect of exposure to a conditioned inhibitory stimulus, is that it results in the retardation of the development of the CR to that stimuli if it is subsequently positively paired to the UCS (Mackintosh, 1983; Rescorla, 1969). However, during the relapse phase, no lower drug preference was observed in the stressed animals of the previous C-I groups as compared to the stressed animals of the C, P-C and P-S groups. Again, these data do not conform to predictions of the conditioning models arguing that a retardation in the re-acquisition of drug SA would be observed in the former C-I groups exposed to paired stress in the relapse phase.

It may be that predictions based on conditioning models were not observed in this experiment because unconditioned factors also were involved in the effect of stress on increased opioid SA and withdrawal. That is, the lack of differences in drug preference between the C-I and the C groups may have resulted from the conditioned inhibition procedure abolishing the unconditioned effect of stress on increased opioid SA.

It is not clear, at present, which unconditioned mechanisms are involved in the effect of stress on increased opioid SA. It may be that the interaction between opioid and other drugs of abuse, and stress at the mesolimbic dopaminergic system may serve as an unconditioned mechanism of the effect of stress on increased opioid SA. This brain system has been implicated in the rewarding effects of a variety of drugs of abuse (Wise & Bozarth 1987; Koob & Bloom 1988). Further, both stress and drugs are known to activate the mesolimbic

dopaminergic system, and cross-sensitization between stress and drugs is observed at the behavioral level (See Kalivas & Stewart 1991 for a review). In addition, Piazza et al. (1991b) recently reported that initiation of amphetamine SA is related to both increased reactivity to a stressor (a novel environment) and increased activity of the mesolimbic dopaminergic system in the nucleus accumbens. Both amphetamines and opioids activate the dopaminergic mesolimbic system. Therefore, it may be that alterations in the mesolimbic system after exposure to stress may contribute to increased opioid SA as well.

Alternatively, it may be that pharmacokinetic factors are involved in the stress-opioid interaction (i.e., stress causes a decrease in the drug bioavailability) (Cohen et al., 1985; Grunberg & Baum, 1985). However, as can be seen in Table 6 and 13, comparable levels of plasma and brain opioid levels were observed in this experiment. Further in a recent study we (Shaham et al., 1992) found that the effect of stress on opioid preference is not associated with decreases in brain or plasma morphine or fentanyl concentrations. Appelbaum and Holtzman (1984) similarly found no differences in plasma and brain morphine levels in rats exposed to 30 min of IM stressor compared to controls. Taken together, these reports indicate that pharmacokinetic factors are not likely to be involved in the stress-opioid interaction.

In summary, the results obtained for morphine and fentanyl, with the exclusion of the conditioned withdrawal measurement during the *Drug-free, stress-free* period and fentanyl withdrawal during *Phase 1*, support the research hypotheses. Exposure to IM stressor increased opioid SA and enhanced naloxone-induced opioid withdrawal. Further, the stress-induced enhancement of morphine and fentanyl SA and morhine withdrawal was dependent upon the temporal relationship between the stressor administration and the drug SA period. That is, IM stressor increased morphine and fentanyl SA and morphine withdrawal when it predicted the drug availability (the P-C and P-S groups), but not when it predicted the absence of the drug (the C-I group) as compared to a control condition of no exposure to stress.

EXPERIMENT 2: EFFECT OF stress ON FENTANYL SELF-ADMINISTRATION USING OPERANT CONDITIONING CHAMBERS

Experiment 2a - Initial experiment

METHODS

Overview

The purpose of this experiment was to determine whether the effect of stress on opioid SA can be generalized to a different experimental setting including a different stressor (electric footshock), and a different behavioral response (lever pressing to obtain the drug). This experiment examined major hypothesis 1 and the corollary hypotheses 6-8. In this experiment, after a period of induction of fentanyl (25 ug/ml) SA in the operant chambers for 1 h a day by water deprivation for 4 weeks (*Initiation Phase*), water was provided ad libitum in the home cage. Electric footshock (0.4-0.8 mA) for 10 min was administered during the *Initiation Phase* so that the stressor administration reliably predicted the drug SA period. During the *Testing Phase* (12 days) animals were tested for lever pressing for fentanyl or water in the operant conditioning chamber while they were either exposed or not exposed to electric footshock prior to the SA period.

Subjects

Eight male Wistar rats, 8-10 weeks old (Charles River), were used as subjects. Animals were kept in the home cage for 23h a day with food (purina rat chow pellets) freely available except for two days prior to the training sessions (see Procedure section). Animals were maintained in a temperature (23^0 C) and humidity (50%)-controlled room. Light-dark was a 12-12 hours cycle (light on 0700-1900), and animals were individually housed.

Drugs

Fentanyl-Hcl (NIDA) in a concentration of 25 ug/ml dissolved in tap water was used. Naloxone-Hcl (Dupont Pharmaceutical) in a concentration of 0.4 mg/ml in 0.86% NaCl (saline) solution injected i.p. was used to precipitate the withdrawal syndrome. Naloxone-Hcl dosage was 1.0 mg/kg. These drug dosages were based on previous studies in our laboratory (Shaham et al., 1992).

Electric footshock stressor

Mild constant current electric footshock was used in this experiment. The shock was administered for 10 min prior to the sessions in the operant chamber. The inescapable shock was administered for 1 sec every 5 sec. Shock intensity was 0.8 mA and it was delivered through a scrambler to the grid of the floor in the operant chamber. Electric footshock was used in order to examine the effect of another commonly used stressor in the animal literature (e.g., Coderre & Rollman, 1984; Kant et al., 1987; Kant et al., 1983; Lewis, Cannon & Liebeskind, 1980; Meyerhoff et al., 1988) on opioid SA. This stressor is known to affect a variety physiological indices of stress (e.g., plasma corticosterone) when administered on an acute or repeated basis (Kant et al., 1987; Meyerhoff et al., 1988). The specific shock parameters used in this experiment were based on Lewis et al. (1980), Coderre and Rollman (1984), and Kant et al. (1983).

Apparatus

Four commercial, sound attenuating, operant conditioning chambers (ENV-001, Med associate Inc., East Fairfield, VT) each equipped with two levers and two 65 ml liquid dispensers (ENV-201) were used. A single operation of the dispensers results in the administration of 0.1 ml of a given solution. The operant chambers were connected to a cabinet and power supply (SG600/C) which in turn was connected via a 16 port interface (DIG-700) to a 386 VGA computer. MED-PC Medstate Notation (Tatham & Zurn, 1989), Turbo Pascal, Quattro-Pro, and SAS softwares were used to operate the operant conditioning chambers, and to record and analyze the data.

Procedure

Animals were handled for the first three days in order to acclimate them to the animal room and to the experimenter. The first training session in the operant chambers started after 48 hours of water deprivation. Animals were shaped for lever pressing for water in the operant chambers for 1 h/day for two days under a fixed ratio-1 schedule of reinforcement (FR-1, each lever response is followed by a reinforcement). For the next 8 days, the FR ratio was gradually increased to an FR-10 schedule of reinforcement.

After the training sessions for water, fentanyl (25 ug/ml) was introduced in the operant chamber under an FR-10 ratio for 1 h/day for 4 weeks. The intermittent schedule was introduced because higher number of responses per session are observed under this schedule for drug SA. During the sessions, the houselight was on during exposure to the footshock stressor, the light above the operative lever (left lever) was on during the drug SA period, and a white noise was turned on for 0.1 sec every time the animal met the schedule requirement. During the non-stress testing period, animals were left in the operant chamber for 10 min prior to the drug SA period while the shock and the houselight were turned off.

Intermittent electric footshock (0.4-0.8 mA, 1 sec on, 4 sec off) was introduced on the first day of the *Initiation Phase* and was administered daily for 10 min/day just prior to the fentanyl SA period. The electric footshock was gradually increased from 0.4 mA to 0.8 mA during the first 12 days of the *Initiation Phase*. During the *Initiation Phase* and the water training period, animals were allowed to drink 8-12 ml/day in their home cage every other day. Otherwise, water was not available in the home cage. Food was continuously available in the home cages.

The *Testing Phase* was conducted after the *Initiation Phase*. During this phase both water and food were continuously available in the home cage. The FR schedule during the *Testing Phase* was reduced to FR-2 because in a pilot study conducted prior to this experiment, some animals did not lever press for the drug when higher schedule requirement were introduced during a testing period. For the first 4 days of the *Testing Phase*, the stressor was not administered in order to determine baseline level of fentanyl consumption for non-water deprived rats. For the next 4 days, electric footshock stressor was introduced just prior to the testing sessions in the operant chambers. Then, both the stressor and drug were removed and water SA was determined for an additional 4 days.

Experimental sessions were conducted for 7 days a week. Withdrawal measures after naloxone (1 mg/kg) administration were assessed 5 days before the end of the *Initiation Phase*. The withdrawal measures were conducted as

previously described (see Methods section Experiment 1).

Statistical and power analyses

Repeated measures ANOVA was used to examine the dependent measure of the number of responses on the operative lever in the operant chambers. Significance level was based on α=0.05. Based on previous publications (see Carroll & Meisch, 1979, 1984), it was estimated that a sample size 8 subjects would be sufficient to detect differences between the stress and non-stress conditions if the stress manipulation would cause an increase in drug SA.

RESULTS and DISCUSSION

All animals learned to lever press for the water and fentanyl solution. In the last week of the *Initiation Phase*, the animals range of responses on the operative lever was 762-1506 responses per session corresponding to about 7.5-15 ml/day of the fentanyl solution (a dosage of approximately 0.6-1.2 mg/kg). In contrast, a very low response rate was observed on the non-operative lever (range 0-17 responses/session). During this week naloxone (1 mg/kg) was injected at the end of one of the sessions. The withdrawal data indicate that despite the short duration of the fentanyl SA, the animals developed physical dependence to the drug during the *Initiation Phase*. Several withdrawal symptoms (presented as group mean±SEM) were observed during the 20 min of withdrawal assessment including teeth chattering (20.0±1.9 counts/20 min), ptosis (1.1±0.5), excessive grooming (8.1±2.0) and abnormal posture (10.2±1.6). The withdrawal symptoms of wet-dog shakes, diarrhea, and body weight loss were not observed.

The stress manipulation, however, was not effective in enhancing drug SA in the operant chambers during the *Testing Phase*. Repeated measures ANOVA did not reveal significant condition effect for the stress+drug (47.4±8.9 responses/session; mean±se), no-stress+drug (45.2±8.9) and no-stress+water conditions (63.5±21.0) [$F(2,14)=1.6$, ns.].

Taken together, the main finding of this experiment was that electric footshock stressor did not cause an increase in the rate of oral fentanyl SA in the operant chambers as compared to a non-stress condition. This finding may indicate that electric footshock stressor does not affect drug SA in this animal

model, or that some parameters of the procedure used in this experiment were not optimal for detecting the effect of stress on oral opioid SA.

Several procedural parameters may be related to the lack of effect of electric footshock stressor on oral fentanyl SA. First, it may be that the amount of fentanyl consumed during the *Testing Phase* (approximately 0.12-0.13 mg/kg/day) which is just above the oral analgesic fentanyl threshold (0.1 mg/kg; Janssen Pharmaceutical data) was too low. Therefore, it was postulated that stress did not affect fentanyl SA during the testing phase because not enough drug was consumed by the animals during this phase.

Second, it may be that the abrupt change from a severe water deprivation in the *Initiation Phase* to a complete water satiation in the testing phase resulted in a lack of effect of stress on drug SA. Specifically, although electric shock stressor reliably predicted the drug SA period, it may be that the water deprivation, a strong interoceptive stimulus, served as a discriminative stimulus for the SA period and overshadowed the relationship between exposure to stress and drug SA. Hence, it is possible that the lack of stress effect on drug SA is related to a state dependent learning phenomenon (Overton, 1991) between the water deprivation condition and the drug effects. In other words, the animals learned that the drug serves as a reinforcer only under conditions of water deprivation and not under conditions of stress. Therefore, in the absence of water deprivation during the testing phase, a similar rate of responding for fentanyl was observed in the stress and non-stress conditions because the stimulus controlling the drug SA (i.e., water deprivation) was removed.

Third, it may be that the schedule of shock administration was not optimal to detect changes in opioid SA after exposure to shock stressor. Specifically, the schedule of shock chosen caused the animals to completely avoid the shock (i.e., the animal learned that shock is administered every 4 sec and they avoided the shock by raising 3 paws above the grid line, thus not exposing to the shock which requires the closing of an electrical circuit between two grids). In other words, the animals were exposed to a controllable and predictable stressor. These two factors are known to attenuate and even to completely abolish the

stress response (Baum et al., 1981; Glass & Singer, 1972; Mason, 1975). Therefore, it may be that the lack of effect of electric shock on drug SA results from the fact that the animals were exposed to a controllable and predictable stress.

Fourth, it may be that the low ratio requirement (FR-2) used in the *Testing Phase* was too low to detect differences between the stress and non-stress conditions. Previous research using the oral SA paradigm demonstrated that the effect of food-deprivation on oral drug SA are more pronounced when a higher schedule requirement is used as compared to a lower schedule requirement (Carroll & Meisch, 1979, 1984).

Finally, it is also possible that the severe water deprivation along with the forced consumption of a slightly bitter solution during the initiation phase served as stressors and masked the potential effects of electric footshock stressor on fentanyl SA. This possibility should be explored in future research by examining the effect of water deprivation on physiological indices of stress (e.g., corticosterone, ACTH and prolactin [Kant et al., 1987]).

Based on the above considerations, several changes in the experimental paradigm, hypothesized to enhance the probability of detecting differences between the stress and non-stress conditions, were made and the experiment was repeated with the exclusion of the water training phase. In the section below the methods, results and a discussion of the follow-up experiment are presented.

Experiment 2b - Follow-up experiment

METHODS

Overview

The purpose of this experiment was to determine whether the effect of stress on opioid SA occurs after several modifications in the experimental procedure of the initial experiment are employed. These modifications included a gradual increase of the drug dosage during the *Initiation Phase*, a change in the shock parameters so that the exposure to the shock stressor would be uncontrollable and unpredictable, conducting the *Initiation Phase* under partial water deprivation, a gradual increase in the amount of water consumed in the home

cage during the *Initiation Phase*, utilizing a complete A-B-A repeated measures design (i.e., repeated exposure to stress, no-stress and stress conditions), increasing duration of the water extinction phase, increasing the schedule requirement during the *Testing Phase* to FR-4, and an addition of another key dependent variable (i.e., assessment of drug SA under a progressive ratio schedule).

In this experiment, after a period of induction of fentanyl (25-100 ug/ml) SA in the operant chambers for 30 min/day by partial water deprivation in the home cage (10-35 ml/day) for 3 weeks, the amount of water consumed in the home cage was gradually increased for additional 7 days until all animals drank less water than the amount available to them (*Initiation Phase*). Electric footshock (0.8 mA, 10-70 sec off, 0.1 sec on) was administered for 10 min during the *Initiation Phase* so that the stressor administration reliably predicted the drug SA period. During the *Testing Phase* (44 days) animals were tested for lever pressing for fentanyl under either an FR-4 or a progressive ratio schedules of reinforcement, and for lever pressing for water under an FR-4 schedule of reinforcement in the operant conditioning chamber while they were either exposed or not exposed to electric footshock prior to the drug SA period.

Subjects

Eight male Wistar rats (14-16 weeks old [Charles River]) that were used in the initial experiment, participated in this experiment. Animals were kept in the home cage for 23 h a day with food freely available. Animals were maintained in a temperature (23^0 C) and humidity (50%)-controlled room. Light-dark was a 12-12 hours cycle (light on 0700-1900), and animals were individually housed. The food used was a Purina rat chow. An additional 5 male Wistar rats (14-16 weeks old [Charles River]) that did not participate in the operant chamber experiment were further used in order to evaluate the effectiveness of the

electric footshock stressor manipulation[23]. These animals were maintained under the above mentioned conditions with food and water available ad-libitum.

Drugs

Fentanyl-HCl (NIDA) in a concentration of 25-100 ug/ml dissolved in tap water was used. Naloxone-HCl (Dupont Pharmaceutical) in a concentration of 0.4 mg/ml in 0.86% NaCl (saline) solution injected i.p. was used to precipitate the withdrawal syndrome. Naloxone-HCl dosage was 1.0 mg/kg.

Electric footshock stressor

Mild constant current electric footshock was used. The shock was administered for 10 min prior to the sessions in the operant chamber. The inescapable shock was administered for 0.2 sec every 40 sec on the average (a variable interval schedule of shock administration with a range 10-70 sec). The shock intensity was 0.8 mA and it was delivered through a scrambler to the grid of the floor in the operant chamber. Unlike the shock procedure used in the initial experiment, the animals did not avoid this schedule of shock presentation. It should also be noted that the shock administration was inescapble, unpredictable, and uncontrollable. These factors are known to result in a more pronounced physiological and behavioral response to a given stressor (Glass & Singer, 1972).

Apparatus

Four commercial, sound attenuating, operant conditioning chambers (ENV-001, Med associate Inc., East Fairfield, VT) each equipped with two levers and two 65 ml liquid dispensers (ENV-201) were used. A single operation of the dispensers results in the administration of 0.1 ml of a given solution. The operant chambers were connected to a cabinet and power supply (SG600/C) which in turn was connected via a 16 port interface (DIG-700) to a 386 VGA computer. MED-PC Medstate Notation, Turbc Pascal, Quattro-Pro, and SAS softwares were used to operate the operant conditioning chambers, and to record and analyze the data.

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[23] The electric footshock stress has never been used in the past in our laboratory. Therefore, it was necessary to examine whether this stressor causes an increase in physiological stress responses in the experimental animals compared with control animals never exposed to either the stressor or fentanyl.

Procedure

An FR-6 schedule of reinforcement was used during the *Initiation Phase* of the drug SA period, and the drug SA sessions were conducted for 30 min. These modifications (i.e., decreasing the duration of the SA session from 60 min to 30 min, and the schedule requirement from FR-10 to FR-6) were made because it was observed in the initial experiment that animals rarely respond for fentanyl for more than 20 min, and it was hypothesized that animals would be likely to continue to self-administer the drug during the *Testing Phase* if more comparable schedule requirements are used in the *Testing* and *Initiation Phases*.

For the first 22 days of the *Initiation Phase*, the drug dosage was gradually increased to 100 ug/ml under a partial water deprivation in the home cage (10-35 ml/day). Because an overall decrease in rate of responding was observed at the highest concentration, the drug concentration was reduced to 75 ug/ml. For the next 7 days the amount of water consumed in the home cage was gradually increased until all animals drank less water than the amount available to them (*Initiation Phase*). Electric footshock was administered for 10 min during the *Initiation Phase* so that the stressor administration reliably predicted the drug SA period. During the sessions, the houselight was on during exposure to electric footshock stressor, the light above the operative lever was on during the drug SA period, and a white noise was turned on for 0.1 sec every time the animal met the schedule requirement. During the non-stress testing period, animals were left in the operant chamber for 10 min prior to the drug SA period while the shock and the houselight were turned off.

During the *Testing Phase* (44 days) animals were tested for lever pressing for fentanyl under either an FR-4 or a progressive ratio in the operant conditioning chamber while they were either exposed or not exposed to electric footshock prior to the SA period. A higher schedule requirement was used in the present experiment because it was suspected that one of the reason that stress did not affect fentanyl SA in the initial experiment was the low (FR-2) schedule requirement used. In the progressive ratio sessions the initial schedule of reinforcement was FR-1, and the schedule requirements were increased by one

response at a time after each successful earning of a drug reinforcement (i.e., increasing the schedule requirement from FR-1 to FR-2, FR-3, FR-4.... FR-n). The *Testing Phase* included 5 days of fentanyl SA (4 days of FR-4 schedule and 1 day of a progressive ratio schedule) + stress exposure prior to the SA period, 5 days of no exposure to stress prior to the SA period (4 days of FR-4 schedule and 1 day of a progressive ratio), and 5 days of re-exposure to fentanyl SA and stress (4 days of FR-4 schedule and 1 day of a progressive ratio schedule). After this time period, water was substituted for the drug and the animals self-administered water under an FR-4 for 4 days under conditions of stress, 4 days of no exposure to stress and an additional 13 days of exposure to stress. In the last 8 days of the experiment, the fentanyl was reintroduced and the animals lever press for drug under an FR-4 ratio under conditions of exposure or no exposure to stress.

Withdrawal measures after naloxone (1 mg/kg) administration were assessed during the *Initiation Phase* at the highest concentration (100 ug/ml). At the end of the experiment animals were exposed to electric footshock and immediately decapitated without anaesthesia. Trunk blood was collected in heparinized tubes (50 ul of heparin/tube) and centrifuged for 20 min at 1500 x g at 4 degrees C. RIA for plasma corticosterone (ICN Biomedic) was performed as described in the Methods section Experiment 1. The RIA for corticosterone also included additional 5 drug-free animals that were not exposed to stress in order to examine the effectiveness of the stress manipulation.

Statistical analyses

Repeated measures ANOVAs were used to examine the dependent measures of the number of responses in the operant chambers under an FR-4 and a progressive ratio schedule of reinforcements. The data within each experimental condition (i.e., stress+drug, no-stress+drug, stress+water, no-stress+water) were averaged across days. *Post hoc* analyses comparing individual time points utilized Fisher Least Square Differences Test. As compared to the Duncan *post hoc* test, the Fisher test is associated with a slight increase in power to detect *post hoc* differences, but with a corresponding slight increase in Type-1 error. This test was chosen for the present experiment for two reasons: The small sample size used

and the fact that it is not possible to compute the Duncan test for *post hoc* repeated measures comparisons using either SAS or SPSSx statistical packages. Significance level was based on alpha=0.05. Additional dependent measures included plasma corticosterone levels, 23 h water consumption in the home cage, rate of responding for the non-operation lever (right lever) in order to assess overall changes in the effect of shock stressor on activity level, and latency for the first and fifth reinforcements in order to examine whether exposure to shock stressor affects the latency for obtaining the drug reinforcement.

RESULTS

Two animals were excluded from the data analyses. One animal earned on the average less than one reinforcement per session during the first 20 days of the *Testing Phase* as compared with a range of 3-40 reinforcers per session earned by the other animals. Another animal consistently did not drink the drug solution after responding on the operative lever, thus it cannot be assumed that its behavior is related to drug SA. Further, because the response in the first day of each experimental condition (i.e., exposure to stress+drug, no-stress+drug, repeated stress+drug, stress+water, no-stress+water etc.) was, in general, deviating from the other 3 days, the data from the transition days were excluded from the analyses. For the second stress+water SA condition that was longer than the other experimental conditions (13 days vs. 4-5 days in the other experimental conditions), the data were collapsed to 4 blocks of 3 days average in order to examine the rate of extinction of lever pressing during this phase. In addition, the drug SA in the first day of the repeated drug condition was reinstated by giving few drops of the fentanyl solution to all rats because an extinction of lever pressing was observed in 3 of the 6 animals by the end of the water sessions. The rate of response during this session was not included in the data analyses.

At the highest concentration (100 ug/ml) during the *Initiation Phase* naloxone (1 mg/kg) was injected at the end of one of the sessions. During this session, the rate of responses ranged from 156-300 responses/session corresponding to about 2.5-5 ml of solution (a dosage range of 0.6-1.2

mg/kg/day). The withdrawal data indicate that despite the short duration of the fentanyl SA, the animals developed physical dependence to the drug during the *Initiation Phase*. Several withdrawal symptoms were observed during the 20 min of the withdrawal assessment including teeth chattering (11.0±3.8, mean±se), ptosis (1.2±1.1), excessive grooming (6.2±2.0) and abnormal posture (3.7±1.5) and wet-dog shakes (0.7±0.4). The withdrawal symptoms of diarrhea and body weight loss were not observed.

Analysis of levels of plasma corticosterone revealed that electric footshock caused a reliable increase in plasma corticosterone levels indicating that the stress manipulation was effective. The difference between the experimental animals exposed to electric footshock stressor (180.3±10.4 ng/ml) prior to their sacrifice in the last day of the experiment vs. 5 control animals that were never exposed to either stress or fentanyl (82.4±27.8) was significant [$F(1,9)=16.7$, $p<0.05$].

Figures 8a and 8b present average rate of response on the operative lever during the *Testing Phase* for the FR-4 ratio schedule conducted during the drug sessions and water sessions, and for the progressive ratio schedule conducted only in the initial drug sessions, respectively. Exposure to shock stressor caused an increase in rate of response for fentanyl for both schedule requirements. Repeated measures ANOVA revealed significant condition effects for the FR-4 schedule and the progressive ratio schedule [$F(10,50)=2.4$, $p<0.05$, and $F(2,10)=5.4$, $p<0.05$, respectively]. *Post hoc* analyses for the FR-4 schedule during the initial drug sessions revealed significant or near significant differences between the first period of exposure to stress and the second period of exposure to stress vs. the non-stress condition [$F(1,5)=5.6$, $p<0.07$, and $F(1,5)=7.6$, $p<0.05$, respectively]. *Post hoc* analyses for the progressive ratio schedule during the first 3 drug sessions (stress+drug, no-stress+drug and stress+drug) revealed significant or near significant differences between the first period of exposure to stress and the second period of exposure to stress vs. the non-stress condition [$F(1,5)=5.9$, $p<0.06$, and $F(1,5)=23.1$, $p<0.05$, respectively].

An extinction of the SA behavior was observed under conditions of stress when fentanyl was substituted by water. Significant or near significant differences were observed in the comparison of the initial 2 stress+drug sessions to the final stress+water session [[$F(1,5)=7.6$, $p<0.06$, and $F(1,5)=4.0$, $p=0.1$, respectively]. Further, exposure to shock stressor did not lead to a non-specific increase in responding on the operative lever. Specifically, no significant stress effect was observed on water SA (i.e., rate of water SA during the no-stress condition was not significantly different from any of the water+stress conditions). In addition, upon substitution of the drug with water, the animals responded by initially increasing their rate of response and subsequently decreasing it (i.e., extinction). Significant or near significant differences were observed between the first exposure to the water+stress condition vs. the first no-stress+drug condition and the last exposure to the water condition [$F(1,5)=5.7$, $p<0.07$, and $F(1,5)=14.6$, $p<0.05$, respectively] (see Figure 8a). Further, though not statistically significant, a trend for increased rate of response was observed in the initial stress+water condition as compared to the first stress+drug conditions.

In the last 8 days of the *Testing Phase*, the fentanyl was reintroduced and rate of response was determined under conditions of exposure to stress and no exposure to stress. During this time period, however, it cannot be concluded that the animals reinstated their drug SA after the long extinction period with water. Significant differences were observed for stress condition [$F(1,6)=6.9$, $p<0.05$] indicating higher drug SA under the stress condition as compared to the non-stress condition. However, no significant differences were observed between the last stress+drug condition and the water conditions or the initial drug conditions.

Examination of individual animals' data for the FR-4 and progressive ratio schedules revealed that 4 of the 6 animals, and 5 of the 6 animals, respectively, increased their drug SA during the two initial stress conditions as compared to the non-stress condition; that lever pressing on the operative lever was lower in the last water session as compared to the initial stress+drug sessions in 5

of the 6 animals; and that all animals increased their drug SA in the last stress+drug condition as compared to the last non-stress+drug condition.

The effect of shock stressor on lever pressing on the operative lever for the drug cannot be explained by a non-specific increase in lever pressing or fluid consumption. That is, no significant experimental condition effect was observed for rate of responding on the non-operative lever and water consumption in the home cage (see Table 14). The shock stressor did not significantly affect the latency for response to the drug (see Table 14).

Finally, a relatively small n was used in this experiment, and large individual differences in lever pressing for drug or water were observed during the *Testing Phase* (e.g., during the initial stress+drug sessions and the first water session the range of responses per session was 13-86 and 9-125, respectively). Therefore, the data were further analyzed by non-parametric methods (i.e., Friedman Two-Way ANOVA and Median Tests for related samples). The non-parametric analyses are not presented because they essentially replicated the results of the parametric statistics (e.g., significant or near significant stress effects were obtained during the drug session for the FR-4 and for the progressive ratio).

DISCUSSION - Follow-up experiment

The results of the initial drug sessions of the follow-up operant conditioning chamber experiment indicate that exposure to footshock stressor caused a significant increase in fentanyl SA as compared to a non-stress control condition. Increased rate of responding after exposure to stress was observed for both FR-4 and progressive ratio schedule of reinforcements. In addition, the data obtained rule out two possible explanations (i.e., stress-induced changes in water balance, and stress-induced non-specific lever pressing) for the effect of stress on oral opioid SA. Specifically, over time, an extinction of the lever pressing response was observed when the drug was substituted with water. In addition, no differences in rate of response between the stress vs. no-stress conditions were observed during the water sessions, and no differences among the experimental conditions were observed for rate of response on the non-operative

lever and water consumption in the home cage. In addition, it was previously mentioned (see Introduction section) that the stress-induced enhancement of opioid SA by noxious stimuli (e.g., electric footshock) may not be related to the reinforcement properties of the opioids but to their analgesic properties. It should be noted, however, that it is very unlikely that the increased fentanyl SA observed in the present experiment was related to the analgesic properties of the drug. Specifically, the electric shock administration preceded the drug SA period; the animals had no access to the drug during exposure to the stressor; and the stressor was administered 23 h after the drug SA period.

The findings of an initial enhanced rate of responding upon substitution of the fentanyl with water following by a subsequent extinction of lever pressing for the drug is in agreement with previous reports from the i.v. opioid SA animal literature. Several authors have reported that when saline is substituted for the opioid drugs, the typical behavioral response is an initial increase rate of responding (e.g., Dworkin et al., 1984; Steinfels et al., 1982; Schuster & Woods, 1968; Stewart, 1984) followed by an extinction of this response (e.g., Steinfels et al., 1982; Stewart, 1984).

An unexpected finding in this experiment is the long duration of water extinction observed under conditions of stress. Previous oral opioid SA studies reported much shorter extinction periods (few days at the most; Carroll & Meisch, 1984). Procedural differences exist between the present experiment and Carroll and Meisch's studies (e.g., the induction of the drug SA behavior, type of aversive environmental event, type of drug). But it may be that the long extinction duration observed in this experiment is related to the fact that the animals were exposed to repeated stressor administration for a long period of time. In other words, it may be that exposure to stress leads to a prolongation of the extinction phase of a given drug-seeking behavior. This elongation of the extinction phase of the opioid use may be related to the increased relapse to opioid use under conditions of stress reported in the human literature (e.g., Chaney et al., 1982; Kosten et al., 1986; Whitehead, 1974). Future studies should be conducted to address this interpretation directly.

In the last 8 days of the *Testing Phase* when fentanyl was reintroduced, significant differences were observed between the stress condition vs. the non-stress condition. However, no significant differences were observed between the last stress+drug condition and the last water condition. In other words, there is no clear indication that the animals resumed their drug SA behavior when fentanyl was reintroduced and it cannot be concluded that the drug served as a reinforcer during this time period. This lack of reinstatement of drug SA after a period of extinction departs from other reports from the opioid SA literature. It is commonly observed that after a period of extinction, animals reinstate their oral or i.v. opioid SA (see Carroll & Meisch, 1984; Stewart et al., 1984). One possible explanation for this discrepancy is the long extinction procedure (3 weeks) utilized in this experiment as compared to much shorter extinction procedures (minutes or hours to few days) used in other published reports (e.g., Carroll & Meisch, 1984; Stewart, 1984).

It is not clear exactly which factors account for the discrepant results between the two operant experiments. Several procedural changes were made in the follow-up experiment based on the results of the initial experiment. These changes included higher drug concentration, different shock parameters so that the exposure to the shock stressor would be uncontrollable and unpredictable, conducting the *Initiation Phase* under a partial water deprivation, a gradual increase in the amount of water consumed in the home cage during the *Initiation Phase*, and an increase in the schedule requirement. Only future research can determine what was the critical factor that is related to the fact that shock stressor enhanced fentanyl SA in this experiment but not in the initial experiment.

The results of the follow-up experiment indicate that the effect of stress on increased opioid SA is not limited to a single behavioral paradigm (oral opioid SA in the home cage, and 15 min/day of IM stressor as in experiment 1), but it can be generalized to a completely different behavioral paradigm (operant lever pressing for fentanyl and 10 min/day of exposure to uncontrollable/unpredictable electric footshock stressor). This observation

suggests that the stress-induced enhancement of opioid SA may be generalized to other types of stressors (e.g., crowding defeat). Further, it indicates that a more broad understanding of the effect of stress on opioid use may gained if other behavioral models of drug abuse (e.g., conditioned place preference, drug discrimination) also would be utilized to examine the stress-opioid interaction (see also General discussion).

GENERAL DISCUSSION

The main findings of the present experiments are that: 1) exposure to stress increased opioid SA and enhanced the opioid withdrawal syndrome, and 2) conditioning factors mediate, in part, the effect of stress on morphine and fentanyl SA and possibly morphine withdrawal. That is, the effect of stress to increase opioid SA or enhance opioid withdrawal is partially dependent upon the learned association between exposure to stress and the drug availability.

The data further indicate that the effect of stress on opioid SA occurs across different phases of the addiction process (i.e., initiation, maintenance, and relapse), with different types of stressors (i.e., IM and electric footshock), and with different types of behavioral response to obtain the opioids (i.e., drinking from bottles in the home cage, and operant lever pressing in operant conditioning chambers).

In addition, comparable rates of drug SA and withdrawal were observed for the conditioned inhibition groups and control groups[24]. Perhaps, both conditioned and unconditioned factors are operative in the effect of stress on opioid SA and withdrawal. Specifically, conditioned factors may operate in synergy with the unconditioned factors in the case of the P-C and P-S groups and thereby result in enhanced opioid SA or opioid withdrawal. Conditioned factors may counteract the effect of the unconditioned factors in the case of the C-I groups and thereby result in no net effect of stress on opioid SA or withdrawal. Therefore, although the present experiments concentrated on the role of conditioning factors in the stress-drug interaction, future research should

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[24] According to conditioning theories a conditioned inhibition procedure should inhibit the expression of a given response below control levels (Mackintosh, 1983; Rescorla, 1969).

examine the role of unconditioned factors in the relationship between stress and opioids as well. In the sections below, future directions of this study and clinical applications are discussed.

Future research

Several avenues of research follow from the findings of these experiments. These lines of research include examination of the effect of other stressors on opioid SA and withdrawal, generalization of the findings to other animal models of drug abuse, examinations of the effect of other learning manipulations on the effect of stress on opioid SA and withdrawal, examination of other behavioral and pharmacological manipulations that may buffer the effect of stress on opioid SA and withdrawal, and exploration of the neurobiological substrate(s) for the unconditioned factors involved in the effect of stress on opioid SA and withdrawal. These research directions may lead to a better understanding of the mechanisms underlying the effect of stress on opioid use, and possibly to more effective treatment methods for opioid abuse among addicts who increase their opioid consumption or relapse to opioids as a consequence of exposure to stress.

The present experiments used IM and footshock stressors because these stressors reliably produce the physiological stress response in rats (Kant et al., 1987; Meyerhoff et al., 1988). However, generalization of the effect of these stressors to human stress is somewhat limited. After all, these are not stressors that are typically part of the human stress experience. There exist in the literature more ecological valid stressors such as a defeat by an aggressive encounter (Miczek et al., 1986) or social crowding (Thiebot, 1977). Therefore, the external validity of the findings of the current experiments would be enhanced if the effect of stress on opioid SA and withdrawal occurs using these other stressors as well.

A logical extension of this line of research is to use the intravenous (i.v) drug self-administration methodology in which a given response (usually lever pressing) is followed by a drug injection (Yokel, 1987). The i.v. SA paradigm eliminates several confounding variables that are operating in the oral SA paradigm, such as the taste of the drug solution and the use of substantial

water or food deprivation in order to initiate drug SA.

In addition to the i.v. drug SA paradigm, two other methods, conditioned place preference and drug discrimination, can be used to examine the stress-opioid interaction. The *conditioned place preference* paradigm is an alternative procedure of the SA paradigm for assessing the reinforcement properties of drug of abuse (Bozarth, 1987; Phillips & Fibiger, 1987). This procedure is based on the findings that the association of distinctive environmental stimuli with a primary reward such as food or drug injection results in an acquired preference for those specific environmental stimuli in the absence of the primary reward (Phillips & Fibiger, 1987). During the past 20 years, numerous studies reported that many of the drugs (including opioids) used to maintain intravenous SA can be used to establish conditioned place preference (Bozarth, 1987; Phillips & Fibiger, 1987). The place preference paradigm was used by Katz et al. (1980) to examine the effect of noise (95 Db) stressor on morphine (1.5 mg/kg i.p) conditioned place preference. In the fourth day of the experiment, after 3 days of baseline preference measurements, four groups of rats were exposed to four conditions in a 2 (morphine/saline) x 2 (noise/no noise) factorial design. On the following day (test day) the 4 groups were tested for place preference in the absence of the stressor and drug. Results showed that exposure to one time noise stressor potentiated the conditioned place preference induced by morphine. However, this study did not measure the stress response, had no exposure to stress during the testing phase, and used a one-time acute stressor only. Nevertheless, these results tentatively suggest that, as in the case of the SA paradigm, stress may enhance the rewarding properties of morphine administration in the place preference paradigm.

Another paradigm that can be used to examine the stress-opioid interaction is the *drug discrimination* method (Colpaert, 1987; Overton, 1991). The rationale for the use of the drug discrimination method is based on the notion that the stimulus properties of drugs as assessed by this method are related to their abuse liability (Colpaert, 1987). In the drug discrimination paradigm, animals are trained to discriminate a given drug dose (training dose) from saline. The

training drug and saline injections serve as discriminative stimuli that signal which of several operant responses (usually lever press for food) will be reinforced. Drugs are believed to be discriminated on the basis of their sensory-perceptual interoceptive effects. Hence, tests for stimulus generalization can determine whether other pharmacological treatments or changes in drug dosages produce discriminative stimulus effects similar to those of the training drug dosage (Colpaert, 1987). This methodology was used to examine the effect of food deprivation (known to cause a reliable increase in i.v. or oral drug SA [Carroll & Meisch, 1984]), on morphine drug discrimination (Gaiardi et al., 1987). In this study animals were trained to discriminate between morphine (10 mg/kg) and saline under conditions of partial food satiation[25] or food deprivation. Results of the testing phase of this study showed that a shift to the left in the dose-response curve was observed for the food-deprived rats as compared to the partially satiated rats. This finding indicates that the food-deprived rats were more sensitive to the stimulus properties of morphine than were partially food deprived animals. It is suggested that, as in the case of food deprivation, a similar shift in the dose-response curve to the left or increased sensitivity to the stimulus properties of opioids will be observed under conditions of exposure to stressors, such as IM or footshock.

One important unresolved issue in the present experiments is the relative role of conditioned and unconditioned factors in the effect of stress on opioid SA and withdrawal. That is, although Experiment 1 suggests that both conditioned and unconditioned factors are operating in the stress-opioid interaction, the relative magnitude of each of these factors cannot be directly estimated based on the experimental groups used. Specifically, all groups were either exposed to excitatory conditioning (the CS predicts the occurrence of the UCS; the P-C and P-S groups) or inhibitory conditioning (the CS predicts the absence of the UCS; the C-I group). As previously argued (see Introduction section), the relative magnitude of the conditioned and unconditioned effects of stress on drug

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[25] The reason that a partial food satiation conditioned was used is that animals do not lever press for food in operant conditioning chambers under conditions of complete food satiation (Gaiardi et al., 1987).

SA can be estimated by including a "truly random pairing" control group (Rescorla, 1967). The reason for this assertion is that the effect of stress on drug SA in the random pairing group allows an estimate of the unconditioned effects of stress on drug SA. Therefore, by comparing the results of the latter group to a paired-stress condition, a direct estimate of the relative role of the conditioned unconditioned factors in the effect of stress on drug SA can be made. It is suggested that the incorporation of a random-pairing controlled group in an experiment examining the effect of stress on i.v. opioid SA in operant conditioning chambers, in which the random pairing schedule of the drug SA and the stressor administration is controlled by a computer program, may yield important information concerning conditioned and unconditioned stress effects on drug SA and withdrawal.

Based on the data obtained for the C-I groups in this study, another research direction also is indicated. Specifically, the lack of increased opioid SA and withdrawal in the C-I groups as compared with the P-C and P-S groups may reflect effects of the conditioned inhibition procedure to abolish the effect of stress on opioid SA and possibly opioid withdrawal. Therefore, it is hypothesized that this procedure may cause a decreased drug SA in subjects previously exposed to conditions in which the stressor predicts drug availability. It would be of interest to examine the effect of other conditioning procedures that may attenuate the relationship between stress and opioid SA and withdrawal. For example, other conditioning procedures known to attenuate the expression of the CR, such as latent inhibition (pre-exposure to the CS in the absence of the US prior to the conditioning trials [Mackintosh, 1983]), may attenuate the effect of stress on drug SA and withdrawal.

Another research question that should be addressed is what factors may decrease the effect of stress on opioid SA. The results of this study suggest that the dissociation of the temporal relationship between the stressor and drug availability may buffer the effects of stress on increased opioid SA. An extension of this line of research may include exploration of other environmental conditions (e.g., housing conditions, alternative reinforcers, type of diet) and

pharmacological manipulations (e.g., clonidine, an α2-adrenergic agonist used to alleviate the opioid withdrawal syndrome [Gold & Kleber, 1979]) that may buffer the effect of stress on opioid SA and withdrawal.

Finally, it was argued that unconditioned factors also are operating in the effect of stress on opioid SA and withdrawal. For example, it was hypothesized that the interaction between opioid and other drugs of abuse, and stress (Kalivas & Stewart, 1991; Piazza et al., 1991b) at the mesolimbic dopaminergic system may serve as an unconditioned mechanism of the effect of stress on increased opioid SA. Therefore, the measurement of dopaminergic activity in mesolimbic brain substrates associated with drug reward (e.g., nucleus accumbens [Koob & Bloom, 1988], and ventral tegmental area [Wise & Bozarth, 1987]) during exposure to stress and opioids SA may be an important extension of the current experiments.

In addition, the activation of the locus coeruleus noradrenergic system during opioid withdrawal has been suggested as one of the main neurochemical mechanisms underlying opioid dependence (Gold & Kleber, 1979; Koob & Bloom, 1988; Rassmussen et al., 1990). This system also is activated by exposure to stress (Redmond & Huang, 1979; Tanaka et al., 1983). Therefore, increased opioid abuse after exposure to stress may result because of the activation of the locus coeruleus noradrenergic system associated with both opioid dependence and stress. In other words, stress may exacerbate the opioid withdrawal syndrome or even elicit a withdrawal-like syndrome after a drug-free period because of its effects on the central noradrenegic system. Therefore, the measurement of noradrenergic activity during a period of withdrawal in the presence or absence of stress may add important information concerning the mechanisms underlying the effect of stress on opioid withdrawal.

At a more speculative level, neurochemical techniques (e.g., electrophysiology, microdialysis) measuring mesolimbic dopaminergic and locus coeruleus adrenergic activity can be used to examine whether behavioral manipulations (e.g., conditioned inhibition) that affect the relationship between stress and opioid SA are associated with distinct changes in these brain systems. For example, this line of research can explore whether the temporal relationship

between exposure to stress and the opioid drug availability not only affects the behavior aspect of the drug-seeking behavior (i.e., the drug SA), but also affects the biochemical neurosubstrates associated with the effect of stress on opioid abuse.

Clinical implications

The results of the present experiments may have clinical applications to the treatment of opioid abuse. The decreased opioid SA in the P-C and the P-S group after the removal of the stressor during *Phase 2*, and in the fentanyl *stress* group during the relapse phase, suggest that when the stressor predicts the drug availability, its removal will decrease drug SA. The clinical implication is that the removal of the stressor or decreasing its aversive effects will decrease drug consumption among individuals who increase their drug consumption under conditions of stress.

Under many conditions, exposure to stressors that are directly related to drug abuse (e.g., unemployment, family difficulties that [Jaffe 1985]) is beyond the control of both the drug addict and the therapist. That is, exposure to the stress *per se*, is not amenable to change. The results of Experiment 1 (i.e., a conditioned inhibition procedure abolished the stress-induced opioid SA and withdrawal) indicate that by systematically dissociating the exposure to stress from the drug availability, the effect of stress on opioid SA may be reduced or even abolished. Therefore, behavioral treatment interventions that first identify the role of the learned association between stress and increased drug use, and then utilize methods (e.g., conditioned inhibition, counterconditioning) to attenuate this learned association may prove useful in decreasing the effect of stress on drug use and relapse.

In summary, the present experiments suggest that exposure to stress caused an increase in opioid SA and enhance the opioid withdrawal syndrome. Moreover, conditioning factors mediate, in part, the effect of stress on morphine and fentanyl SA and possibly morphine withdrawal. That is, the effect of stress to increase opioid SA or enhance opioid withdrawal is dependent upon the learned association between exposure to stress and the drug availability. Further, the

data indicate that the effect of stress on opioid SA is generalized across the different phases of the addiction process, to different types of stressors, and to different types of behavioral response to obtain the opioids. Finally, a plausible interpretation of the results obtained for the stressed groups is that both conditioned and unconditioned factors are operating in the effect of stress on opioid SA and withdrawal. A continuation of this line of research in the elucidation of the multiple mechanisms involved in the stress-opioid interaction may not only prove useful from a basic science point of view, but may also have clinical application in the treatment of opioid addicts in chronic stress situations. Further, these mechanisms may be generalized to explain the relationship between stress and other drugs of abuse (e.g., cocaine, amphetamines).

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Study	Sample and design	Major results	Methodological considerations
Khantzian et al. (1974)	Heroin addicts; n=5; retrospective case reports.	Patients report that heroin is being used to alleviate stress and other psychological difficulties.	Small n; lack of use of validated questionnaires; and reliance solely on psychiatric interview.
Whitehead (1974)	Opioid addicts; n=16; two year longitudinal study; examination of opioid withdrawal syndrome in patients during methadone treatment.	Stressful life events are positively associated with precipitation of withdrawal symptoms during methadone maintenance.	Measurement of life stress was based on a subjective psychiatric interview.
Rahe et al. (1975)	Heroin addicts; n=50; comparison of life change and illness in heroin addicts who enrolled to treatment vs. medical patients and normal controls; use of the Social Readjustment Rating Questionnaire (SRRQ) and the Schedule of Recent Events (SRE).	Opioid addicts obtained higher scores in the SRRQ and SRE questionnaires in comparison to normal controls and medical patients.	Methodological limitations associated with the use of retrospective life event questionnaires (see text). Control groups were not matched with the heroin addict group.
Robbins (1977)	Heroin addicts during methadone treatment; n=34; a correlational study that examined the relationship between self-reports obtained from a psychological diary and methadone dosage.	Higher doses of methadone were negatively related to anxiety (r=-0.43) and interpersonal stress (r=-0.53).	Validity of the measurements used to assess stress; lack of experimental control: Subjects were not randomly assigned to the experimental conditions (i.e., methadone levels).
Prusoff et al. (1977)	Heroin addicts during methadone treatment; n=106; a correlational study that examined the relationship between life events and depression among methadone patients; use of a modification of Recent Life Events (RLE) questionnaire.	In general, methadone maintained patients reported six times more negative life events than normal controls, and more than twice than depressed patients not maintained on methadone.	Methodological limitations associated with the use of retrospective life event questionnaires (see text).
Krueger (1982)	Opioid addicts during methadone treatment; n=270; case-control study that examined the relationship between stressful life events, depression and relapse to heroin; controlling for baseline levels of life events and comparing patients who relapsed to heroin vs. those who did not; use of the Social Readjustment Rating Scale (SRRS).	SRRS scores of patients who relapsed to heroin (n=48) were 227% higher than the scores of patients who did not relapse to heroin.	Sound methodology; again problems associated with the use of life event questionnaires (see text).
Chaney et al. (1982)	Opioid addicts on methadone maintenance; n=39; a correlational study examining intra- and interpersonal determinants of relapse.	Twenty four percent of relapse episodes were related to negative emotional states associated with stress (i.e., nervousness and anxiety).	The validity of the questionnaires used in the study is not specified; lack of assessment of the relapse determinants over time.
Stimmel & [illegible] (1982)	Heroin addicts; n=128; 10 years follow-up of addicts attending heroin clinics in Israel; comparison of demographic and life-style characteristics of subjects who were abstinent from heroin vs. those who were not at the end of the 10 years period.	Higher rate of abstinence from heroin was observed among heroin patients with "chaotic" life-style in comparison to those with "stable" life-style.	No standardized psychometric scales were used in the assessment of life stress.
[illegible] et al. (1982)	Opioid addicts; n=64; comparison of the psychiatric and social profile of opioid addicts from three different typology types based on the assessment of key events associated with the initiation of drug use.	Initial drug use was associated with traumatic life events in only 31% of the sample.	No standardized psychometric scales were used in the assessment of life stress.
Kosten et al. (1983)	Opioid addicts; initial RLE; prospective study of the relationship between RLE, depression and treatment outcomes.	RLE test scores of the opioid addicts were 185% higher than the scores of depressive patients, and 573% higher than normal controls. RLE scores were positively related to illicit drug use.	Control groups were not matched with the opioid addicts group.
Kosten et al. (1986)	Opioid abuse patients during treatment; n=268; 2.5 years longitudinal study assessing the effectiveness of different treatment modalities in preventing relapse to illicit opioid use; the study further assessed the relationship between depression, life crises and drug abstinence; use of the RLE schedule to assess life crises.	Positive relationship between RLE scores and relapse to illicit opioid use.	Despite the sound methodology no causal relationship between life crises and relapse to opioid use can be inferred.

continued)

Grey *et al.* (1986)	Naltrexone and methadone maintenance treatments outpatients; n=60; a 3 month study examining the relationship between pretreatment life stress as measured by the Daily Hassles Scale, somatization, and social support with the treatment outcomes of degree of drug abuse and retention rate.	In the methadone patients, drug abuse was associated with the presence of somatic symptoms and increased stress. In the naltrexone patients, somatic symptoms and stress were associated with dropout from treatment.	Short duration of assessment; the stress measurement (daily hassles) was taken only before treatment and not during the 12 weeks of the study. The latter assessment could have provided stronger support to the research hypothesis due to closer temporal relationship between stress and relapse.
Hall *et al.* (1990)	Alcoholics, opioid addicts and cigarette smokers; n=221 (72 opioid addicts); subjects were followed for 12 weeks after the termination of treatment; stress measurement included a life event questionnaire, mood states and a daily hassles questionnaire.	Stress was only associated with relapse in the retrospective assessment (level of stress after the occurrence of the relapse). Level of stress was not related to relapse in the prospective assessment (level of stress prior to the relapse episode).	Sound methodology.
O'Doherty (1991)	Alcoholics, opioid addicts and cigarette smokers recruited from a community based drug clinic; n=103 (31 opioid addicts); use of a matched control group; subjects were assessed every 3 months for a period of 18 months; stress level was measured by a life event questionnaire constructed by the author.	Heroin addicts reported more stressful life events than their matched control group. But, the differences between the above mentioned groups were found to consist largely of events that were the consequences of the drug use itself.	The first study that included an appropriate control group of drug-free subjects matched for sociodemographic characteristics.

Table 2: Average morphine consumption data and dosage levels in the experimental groups during *Phase 1* of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Dosage level in the last week (mg/kg/day)	32.4±1.0	34.9±1.4	32.9±1.1	34.6±1.6
Baseline water (6 h/day) consumption (ml/day)	29.1±1.3	26.4±2.3	28.9±2.3	27.8±1.4
Drug consumption during forced consumption days (ml/day)	28.1±0.8	30.3±1.1	30.4±1.1	30.8±1.2
Baseline choice drug consumption (ml/day)	4.6±0.3	5.9±0.3	4.6±0.2	5.0±0.4
Baseline choice drug preference (%)	14.9±1.2	18.4±1.2	14.1±0.7	17.0±1.6
Water consumption during *Non-test choice days* (ml/day)	18.1±1.2	15.6±0.4	17.7±1.9	16.8±1.1
Drug consumption during *Non-test choice days* (ml/day)	7.5±0.8	10.3±1.0	7.2±0.8	12.1±1.5 [1,2]
Drug preference during *Non-test choice days* (%)	29.3±2.8	38.2±2.7 [1,2]	29.8±3.1	41.1±3.3 [1,2]
Water consumption during *Test choice days* (ml/day)	16.3±0.9	14.1±1.1	18.2±1.7	16.2±1.1
Drug consumption during *Test choice days* (ml/days)	8.2±1.1	13.3±1.6 [1,2]	7.8±1.2	13.3±2.2 [1,2]
Drug preference during *Test choice days* (%)	31.5±3.8	46.3±4.3 [1,2]	29.7±4.2	42.8±4.6 [1,2]

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
2 - Significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.05$.

Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 3: Morphine withdrawal symptoms after naloxone injection in the experimental groups - *Phase 1* of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Diarrhea episodes (# / 20 min)	1.0±0.6	2.0±0.7	1.2±0.7	2.8±0.6
Teeth chattering (# / 20 min)	5.7±1.7	11.6±2.7	6.9±1.3	16.0±4.5 [1,2]
Excessive grooming (# / 20 min)	1.8±0.7	4.4±1.5	2.0±0.8	4.0±1.0
Abnormal posture (# / 20 min)	4.7±1.8	13.5±3.7	6.4±2.3	9.5±3.2
Total withdrawal score (# / 20 min)	14.4±3.9	35.1±7.4 [1,2]	19.0±3.4	34.0±7.1 [1,2*]
Body weight change (g / 25 min)	1.6±0.5	2.9±0.4	1.7±0.3	2.0±0.5

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
1* - Approaching significant difference from Control group, Duncan *post hoc* test, $p<0.1$.
2 - Significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.05$.
2* - Approaching significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.1$.

Table 4: Average morphine consumption data and dosage levels in the experimental groups during *Phase 2* of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Dosage level in the last week (mg/kg/day)	32.3±1.6	35.7±1.5	33.9±1.4	35.6±1.2
Drug consumption during forced consumption days (ml/day)	27.5±1.2	31.6±1.4	29.9±1.1	29.9±1.4
Water consumption during choice days (ml/day)	13.7±1.4	15.4±0.9	16.5±1.4	14.7±1.3
Drug consumption during choice days (ml/day)	11.3±1.9	17.9±2.6	12.0±2.0	12.4±2.3
Drug preference during choice days (%)	44.1±6.2	51.4±4.5	40.8±5.2	43.7±5.6
Water consumption (6 h/day) in the first week after *Phase 2* (ml/day)	28.1±0.3	28.4±1.2	27.9±0.8	29.3±1.1

Table 5: Withdrawal symptoms after exposure to IM stressor in the morphine groups during stress-free and drug-free period of experiment 1 (+SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Diarrhea episodes (# / 20 min)	0.4±0.3	1.0±0.3	0.4±0.2	0.6±0.3
Teeth chattering (# / 20 min)	0.2±0.2	2.0±2.0	0.4±0.2	1.6±0.6
Excessive grooming (# / 20 min)	3.8±0.7	6.2±2.0	4.4±1.4	9.4±2.1
Abnormal posture (# / 20 min)	0.0±0.0	0.0±0.0	0.2±0.2	0.3±0.2
Total withdrawal score (# / 20 min)	4.4±0.8	9.2±3.3	5.4±1.8	11.9±2.0
Body weight change (g / 25 min)	1.3±0.9	2.4±1.1	1.6±0.8	2.5±0.8

Table 6: Average morphine consumption data, dosage levels, plasma corticosterone, and plasma and brain morphine levels in the experimental groups during *Phase 3* ("relapse phase") of experiment 1 (±SEM)

Measure \ Group	Control (n=17)	Stress (n=16)
Dosage level in the last week (mg/kg/day)	28.1±0.9	26.8±1.0
Baseline water (6 h/day) consumption (ml/day)	30.5±1.0	30.3±0.9
Drug consumption during forced consumption days of the stress period (ml/day)	28.8±0.9	28.2±0.7
Baseline choice drug consumption (ml/day)	7.7±1.9	6.8±0.8
Baseline choice drug preference (%)	20.6±4.0	19.6±4.1
Water consumption during choice days of the stress period (ml/day)	16.7±1.2	18.7±1.3
Drug consumption during choice days of the stress period (ml/day)	9.7±1.1	12.1±1.2
Drug preference during choice days of the stress period (%)	34.2±2.9	38.7±3.3
Drug preference during the last choice day (no stress) (%)	48.5±4.3	40.1±3.9
Plasma corticosterone levels (ng/ml)	44.5±6.9	260.6±27.7 [1]
Plasma morphine levels (ng/ml)	160.8±25.0	166.1±13.9
Brain morphine levels (ng/g)	16.1±3.8	17.0±2.9

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 7: Morphine withdrawal symptoms after naloxone injection in the experimental groups - *Phase 3* ("relapse phase") of experiment 1 (±SEM)

Measure \ Group	Control (n=17)	Stress (n=16)
Wet-dog shakes (# / 20 min)	0.0±0.0	00.06±0.06
Diarrhea episodes (# / 20 min)	0.2±0.1	1.4±0.5 [1]
Teeth chattering (# / 20 min)	6.1±2.1	10.6±2.7
Ptosis (# / 20 min)	0.8±0.5	3.2±1.4
Excessive grooming (# / 20 min)	2.3±0.6	3.0±0.9
Abnormal posture (# / 20 min)	3.8±1.3	10.8±3.3 [1]
Total withdrawal score (# / 20 min)	13.2±3.3	29.1±6.5 [1]
Body weight change (g / 25 min)	0.6±0.3	2.6±0.7 [1]

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.

Table 8: Average fentanyl consumption data and dosage levels in the experimental groups during *Phase 1* of experiment 1 (+SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Dosage level in the last week (mg/kg/day)	2.1±0.1	2.4±0.3	2.5±0.2	2.1±0.3
Baseline water (6 h/day) consumption (ml/day)	30.0±1.1	29.0±1.5	27.6±0.9	29.1±1.5
Drug consumption during forced consumption days (ml/day)	37.1±1.9	43.2±3.5	43.5±1.6	37.7±3.6
Baseline choice drug consumption (ml/day)	15.3±2.6	16.2±3.5	15.9±2.9	15.7±4.3
Baseline choice drug preference (%)	40.0±6.8	43.2±7.6	43.9±8.3	37.0±7.3
Water consumption during *Non-test choice days* (ml/day)	19.4±1.8	19.7±2.5 [2*]	24.7±2.4 [1*]	14.9±1.0 [1*,2]
Drug consumption during *Non-test choice days* (ml/day)	17.4±1.2	23.3±3.6	24.2±2.0	25.0±3.8 [1*]
Drug preference during *Non-test choice days* (%)	47.3±3.1	52.5±5.3	47.9±3.8	60.1±3.9 [1,2]
Water consumption during *Test choice days* (ml/day)	20.8±1.6	18.6±2.1 [2]	25.7±1.7	15.5±1.1 [1*,2]
Drug consumption during *Test choice days* (ml/days)	16.1±0.9	25.1±4.5	21.1±2.3	25.9±4.4
Drug preference during *Test choice days* (%)	43.3±2.7	55.5±5.3 [1,2*]	44.4±4.4	56.9±3.8 [1,2]

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
1* - Approaching significant difference from Control group, Duncan *post hoc* test, $p<0.1$.
2 - Significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.05$.
2* - Approaching significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.1$.
Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 9: Fentanyl withdrawal symptoms after naloxone injection in the experimental groups - *Phase 1* of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Diarrhea episodes (# / 20 min)	1.1±0.4	1.6±0.8	0.9±0.4	1.8±0.5
Teeth chattering (# / 20 min)	12.2±2.6	15.9±2.5	10.1±3.8	19.5±4.4
Excessive grooming (# / 20 min)	3.3±0.9	8.8±2.5	5.5±2.0	6.8±2.1
Abnormal posture (# / 20 min)	5.3±1.5	5.0±1.2	4.7±1.1	6.8±1.6
Total withdrawal score (# / 20 min)	22.0±3.9	31.7±4.6	21.6±5.8	35.2±6.0
Body weight change (g / 25 min)	2.6±1.3	4.0±1.4	2.4±0.8	5.6±1.6

Table 10: Average fentanyl consumption data and dosage levels in the experimental groups during *Phase 2* of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Dosage level in the last week (mg/kg/day)	2.0±0.1	2.2±0.2	2.5±0.2	2.1±0.3
Drug consumption during forced consumption days (ml/day)	37.7±1.8	41.4±4.3 [1,2]	48.1±2.6 [1]	38.5±5.1 [2]
Water consumption during choice days (ml/day)	23.3±1.8	19.4±2.7	23.0±1.3	19.7±2.1
Drug consumption during choice days (ml/day)	19.6±0.9	25.7±5.2 [1*,2*]	31.8±2.5 [1]	21.5±4.6 [2]
Drug preference during choice days (%)	45.0±2.0	53.5±5.6	57.2±1.9 [1]	47.1±5.3 [2]
Water consumption (6 h/day) in the first week after *Phase 2* (ml/day)	33.3±1.0	31.7±2.0	35.5±2.0	31.6±1.3

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
1* - Approaching significant different from Control group, Duncan *post hoc* test, $p<0.1$.
2 - Significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.05$.
2* - Approaching significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.1$.
Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 10: Average fentanyl consumption data and dosage levels in the experimental groups during *Phase 2* of experiment 1 (+SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Dosage level in the last week (mg/kg/day)	2.0±0.1	2.2±0.2	2.5±0.2	2.1±0.3
Drug consumption during forced consumption days (ml/day)	37.7±1.8	41.4±4.3 [1,2]	48.1±2.6 [1]	38.5±5.1 [2]
Water consumption during choice days (ml/day)	23.3±1.8	19.4±2.7	23.0±1.3	19.7±2.1
Drug consumption during choice days (ml/day)	19.6±0.9	25.7±5.2 [1*,2*]	31.8±2.5 [1]	21.5±4.6 [2]
Drug preference during choice days (%)	45.0±2.0	53.5±5.6	57.2±1.9 [1]	47.1±5.3 [2]
Water consumption (6 h/day) in the first week after *Phase 2* (ml/day)	33.3±1.0	31.7±2.0	35.5±2.0	31.6±1.3

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
1* - Approaching significant different from Control group, Duncan *post hoc* test, $p<0.1$.
2 - Significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.05$.
2* - Approaching significant difference from Conditioned-Inhibition group, Duncan *post hoc* test, $p<0.1$.
Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 11: Withdrawal symptoms after exposure to IM stressor in the fentanyl groups during stress-free and drug-free period of experiment 1 (±SEM)

Measure \ Group	Control (n=9)	Partial-Conditioning (n=8)	Conditioned-Inhibition (n=8)	Paired-Stress (n=8)
Diarrhea episodes (# / 20 min)	0.4±0.3	1.1±0.5	0.3±0.2	0.5±0.3
Teeth chattering (# / 20 min)	0.4±0.2	0.6±0.4	0.0±0.0	0.3±0.3
Excessive grooming (# / 20 min)	4.7±0.8	2.3±1.0	2.8±0.7	3.2±0.9
Abnormal posture (# / 20 min)	0.1±0.1	0.0±0.0	0.0±0.2	0.0±0.2
Total withdrawal score (# / 20 min)	5.7±0.7	4.4±1.5	3.0±0.7	4.0±0.9
Body weight change (g / 25 min)	2.2±0.5	2.9±0.7	1.9±0.3	2.0±0.5

Table 12: Average fentanyl consumption data, dosage levels, plasma corticosterone, and plasma and brain morphine levels in the experimental groups during *Phase 3* ("relapse phase") of experiment 1 (+SEM)

Measure \ Group	Control (n=17)	Stress (n=16)
Dosage level in the last week (mg/kg/day)	1.9±0.1	1.8±0.1
Baseline water (6 h/day) consumption (ml/day)	32.4±0.3	33.8±0.4
Drug consumption during forced consumption days of the stress period (ml/day)	43.7±2.6	42.0±2.8
Baseline choice drug consumption (ml/day)	26.2±4.5	24.6±2.8
Baseline choice drug preference (%)	47.7±6.0	44.9±4.4
Water consumption during choice days of the stress period (ml/day)	20.6±1.3	14.6±1.0 [1]
Drug consumption during choice days of the stress period (ml/day)	21.4±3.2	25.7±2.9
Drug preference during choice days of the stress period (%)	46.4±3.9	61.0±3.0 [1]
Drug preference during the last choice day (no stress) (%)	49.9±5.0	52.4±4.5
Plasma corticosterone levels (ng/ml)	85.6±10.1	212.3±22.9 [1]
Plasma fentanyl levels (ng/ml)	6.4±0.5	7.1±0.5
Brain fentanyl levels (ng/g)	5.8±0.9	5.6±0.7

1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.
Note: Statistical analyses for this Table were conducted for the variables average across time. See text for the results of the repeated measures analyses.

Table 13: Fentanyl withdrawal symptoms after naloxone injection in the experimental groups - *Phase 3* ("relapse phase") of experiment 1 (±SEM)

Measure \ Group	Control (n=17)	Stress (n=16)
Wet-dog shakes (# / 20 min)	0.6±0.4	4.3±1.4 [1]
Diarrhea episodes (# / 20 min)	0.4±0.2	0.9±0.3
Teeth chattering (# / 20 min)	6.8±1.9	13.2±1.9 [1]
Ptosis (# / 20 min)	0.9±0.4	1.2±0.4
Excessive grooming (# / 20 min)	5.2±1.0	7.7±0.9
Abnormal posture (# / 20 min)	2.2±0.6	3.2±0.6
Total withdrawal score (# / 20 min)	16.0±3.5	30.5±3.1 [1]
Body weight change (g / 25 min)	2.8±0.6	6.6±1.0 [1]

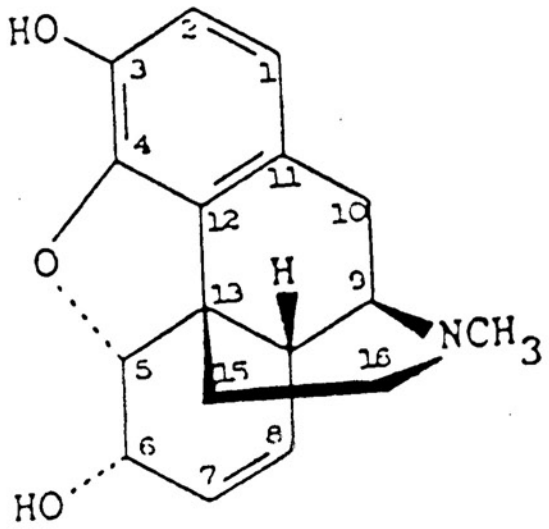
1 - Significant difference from Control group, Duncan *post hoc* test, $p<0.05$.

Table 14: Average right lever (non-operative lever) responses, latency for the first and fifth reinforcements and home cage daily water consumption during the first part of the testing period of experiment 2 (±SEM)

Measure \ Phase	Stress + Fentanyl (3 days average)	No-Stress + Fentanyl (3 days average)	Stress + Fentanyl (3 days average)	First Stress + Water period (3 days average)
Right lever (non-operative lever) responses (# / 30 min)	3.2±1.1	2.6±1.3	2.4±0.7	4.9±1.5
Latency for the first reinforcement (sec)	128.9±38.5	185.2±95.7	208.89±67.7	490.8±187.8
Latency for the fifth reinforcement (sec)	975.4±282.8	1292.3±180.6	989.8±242.3	1236.7±291.9
Daily water consumption in the home cage (ml/day)	56.1±1.7	61.6±9.2	58.4±6.4	60.6±8.9

Figure 1. Chemical structure of morphine and fentanyl

Morphine



Fentanyl

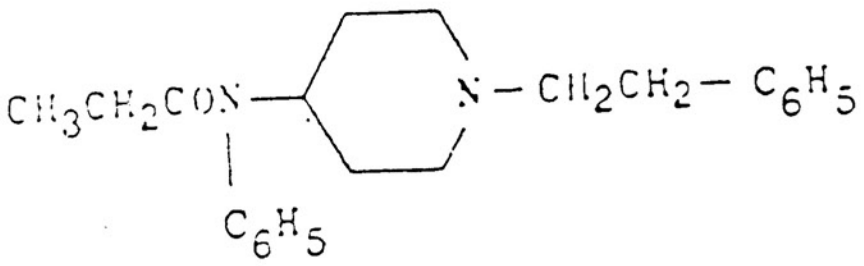
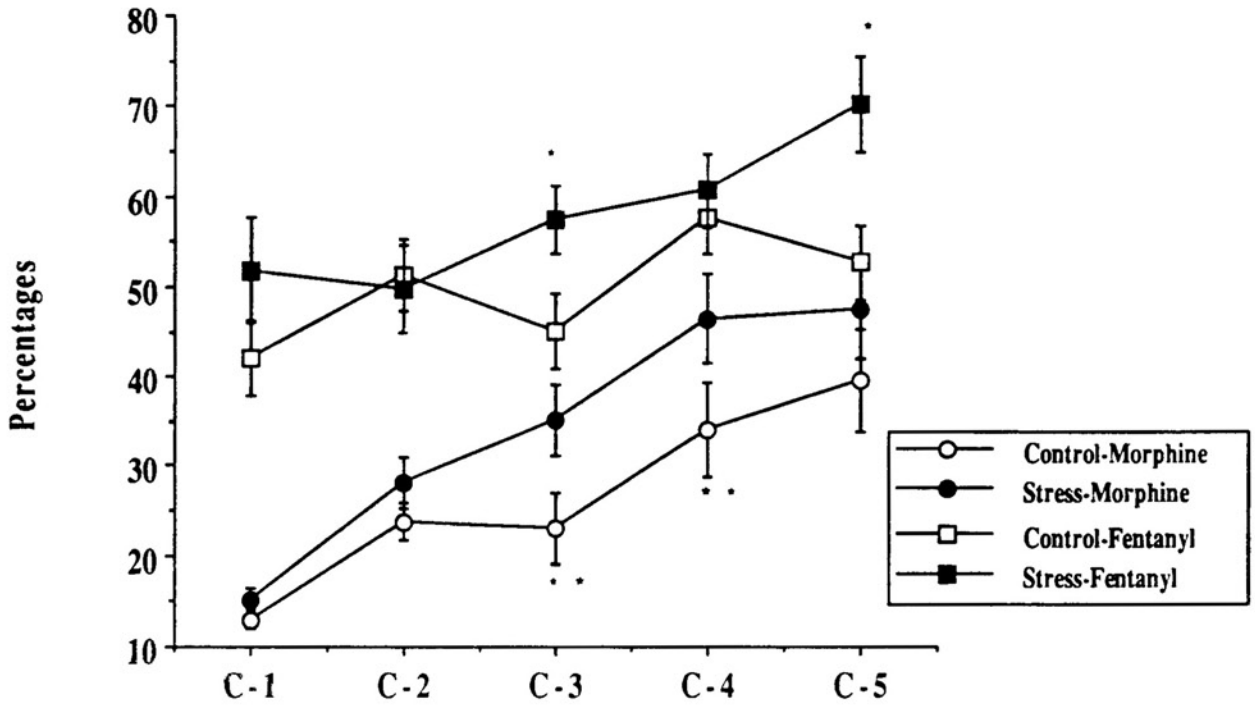


Figure 2a. Morphine and fentanyl preference in the controls and stress groups
(Shaham et al. (1992), PBB, 41, 615-619)



C - Choice; * Significant differences between control-fentanyl and stress-fentanyl groups, $p<0.05$
** Significant differences between control-morphine and stress-morphine groups, $p<0.05$

Figure 2b. Morphine preference during choice days 1 and 11 in control and stress groups

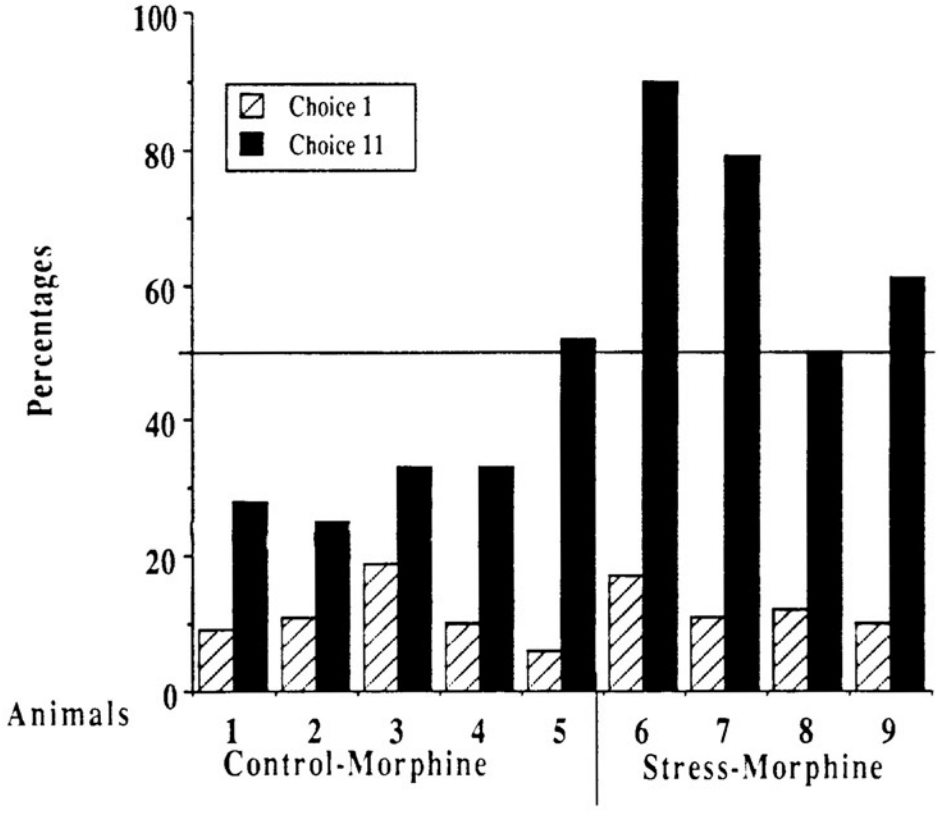


Figure 3. Timeline of Experiment 1

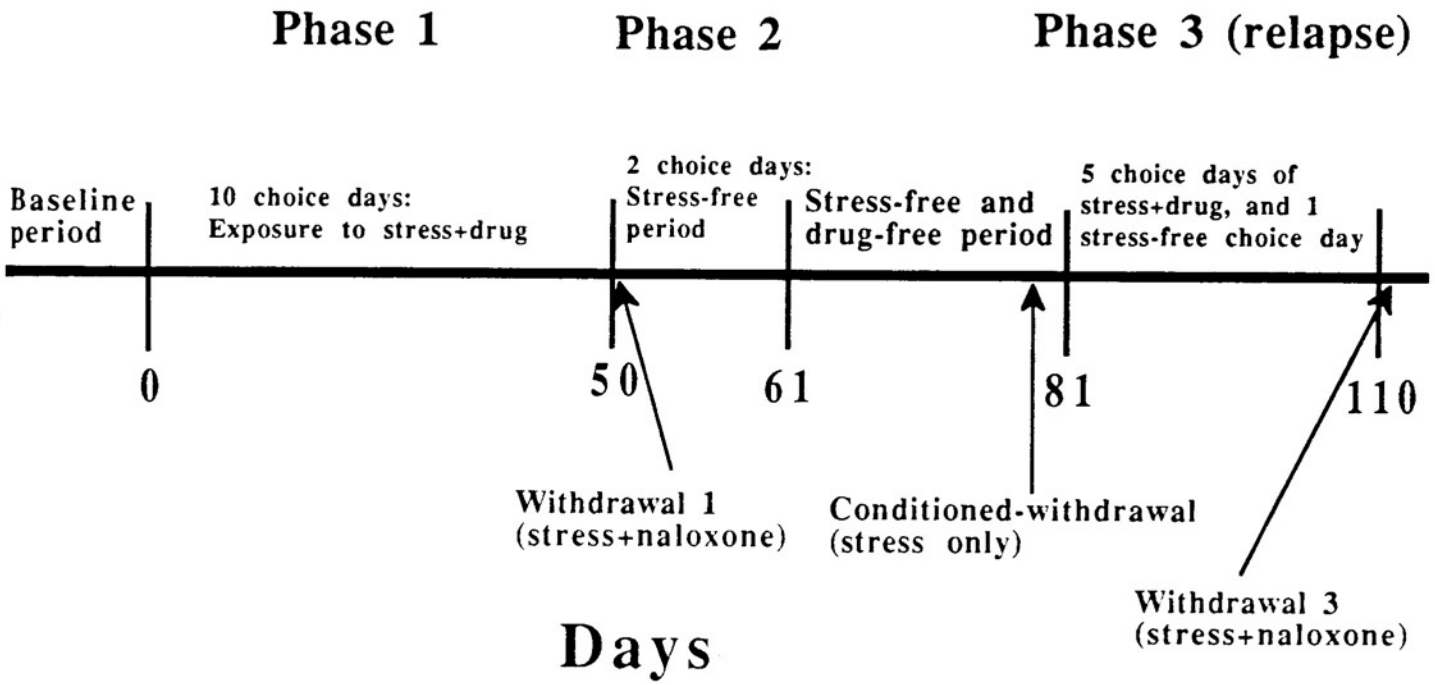
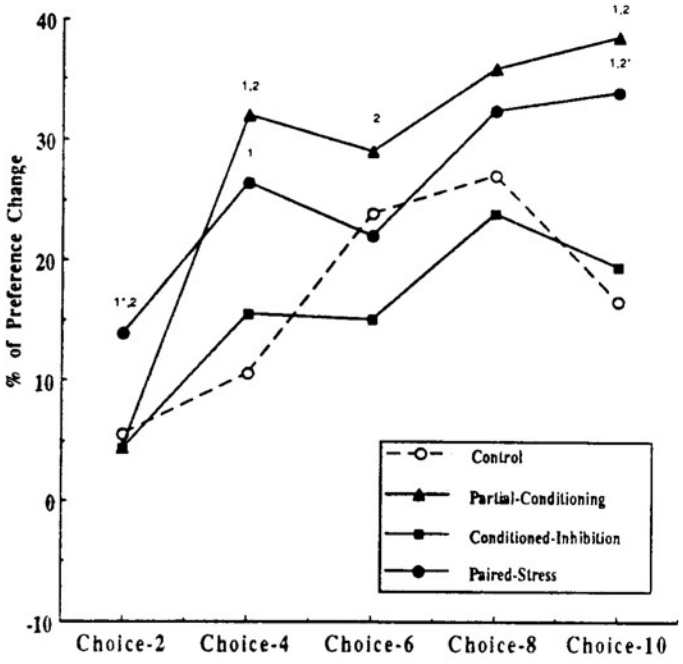


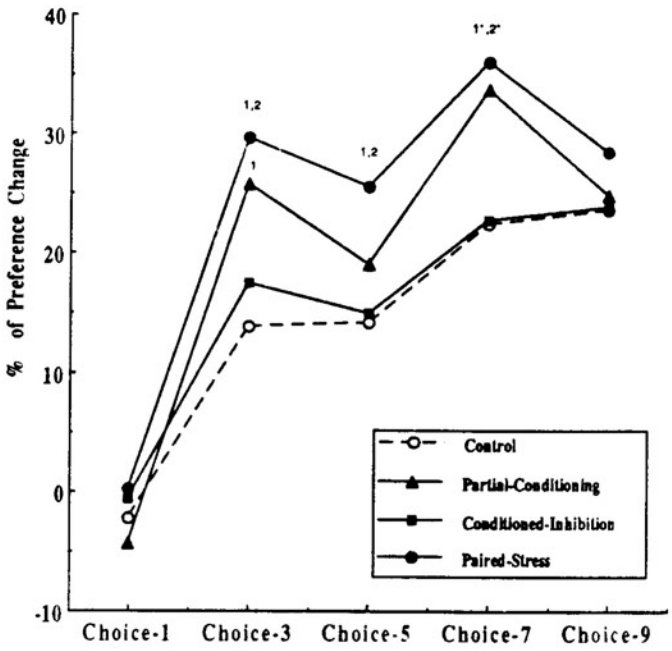
Figure 4a. Morphine: Percent of preference change from baseline:
Test Choice Days



1 - Significant differences from Control group, p<0.05.
1* - Approaching significant differences from Control group, p<0.1.

2 - Significant differences from Conditioned-Inhibition group, p<0.05.
2* - Approaching significant differences from Conditioned-Inhibition group, p<0.1.

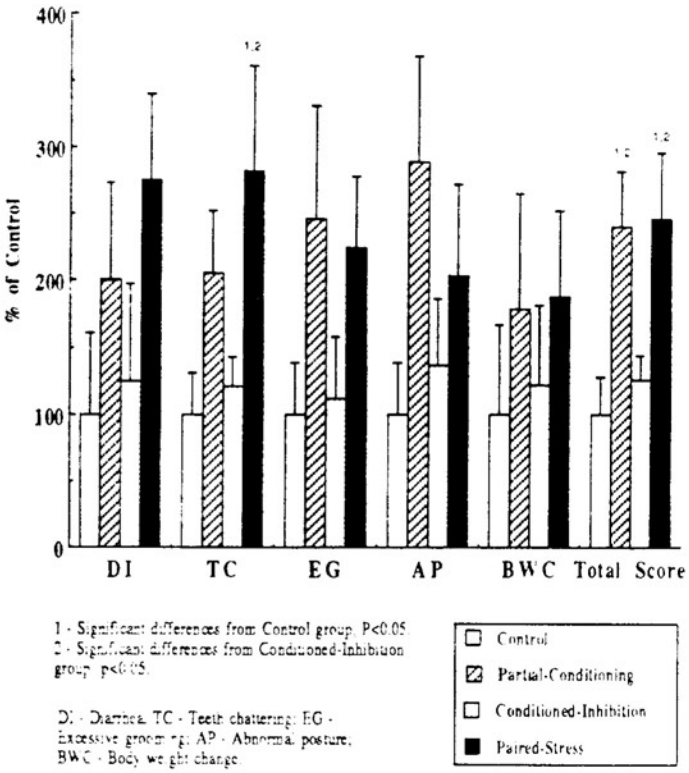
Figure 4b. Morphine: Percent of preference change from baseline:
Non-Test Choice Days



1 - Significant differences from Control group, p<0.05.
1* - Approaching significant differences from Control group, p<0.1.

2 - Significant differences from Conditioned-Inhibition group, p<0.05.
2* - Approaching significant differences from Conditioned-Inhibition group, p<0.1.

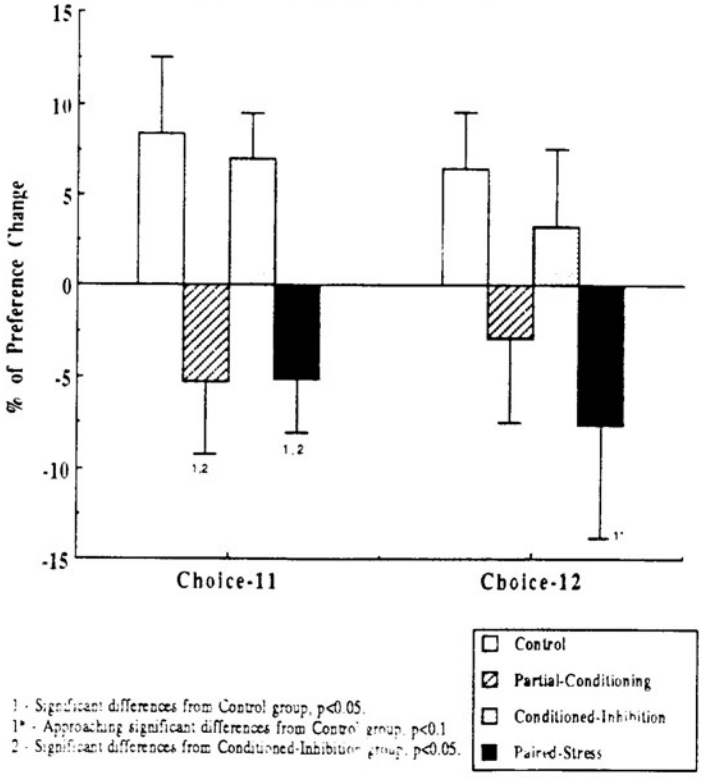
Figure 4c. Morphine withdrawal syndrome



1 - Significant differences from Control group, P<0.05.
2 - Significant differences from Conditioned-Inhibition group, p<0.05.

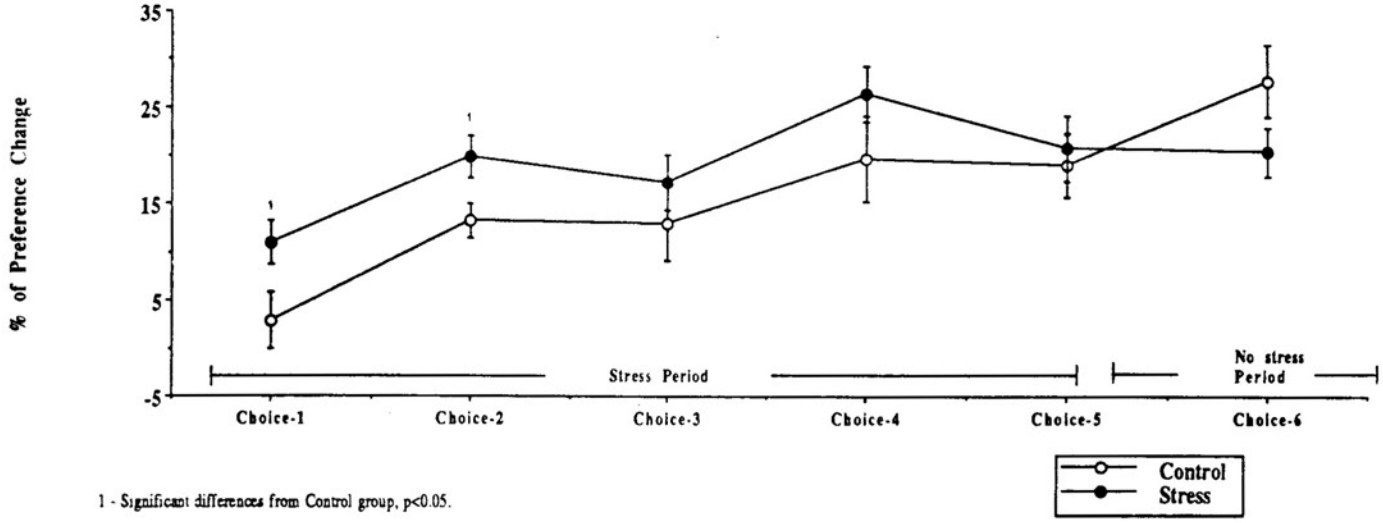
DI - Diarrhea, TC - Teeth chattering, EG - Excessive grooming, AP - Abnormal posture, BWC - Body weight change.

Figure 4d. Morphine: Percent of preference change from stress period:
Phase 2 (no exposure to stress)



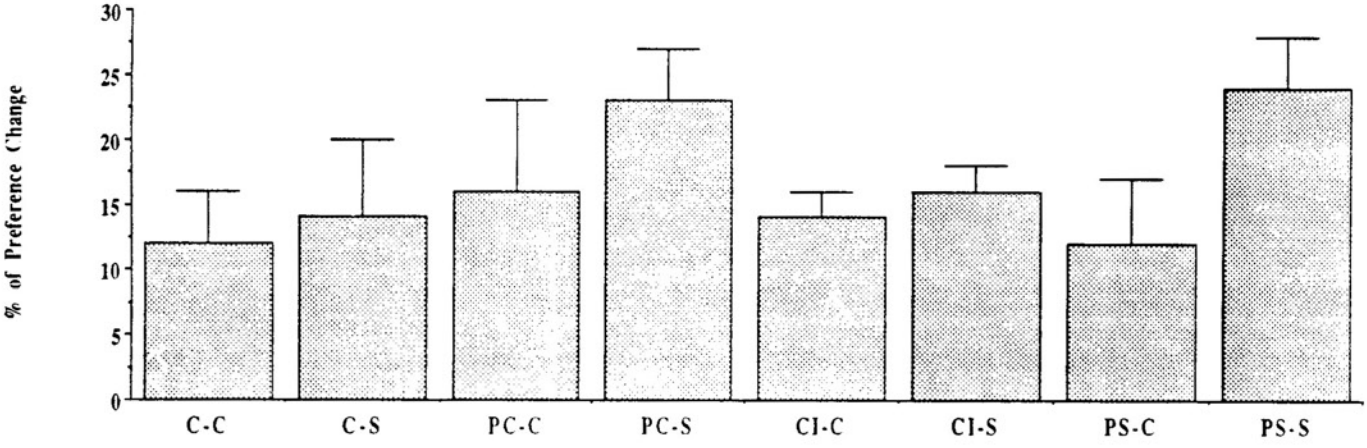
1 - Significant differences from Control group, p<0.05.
1* - Approaching significant differences from Control group, p<0.1
2 - Significant differences from Conditioned-Inhibition group, p<0.05.

Figure 5a. Morphine: Percent of preference change from baseline:
Relapse phase



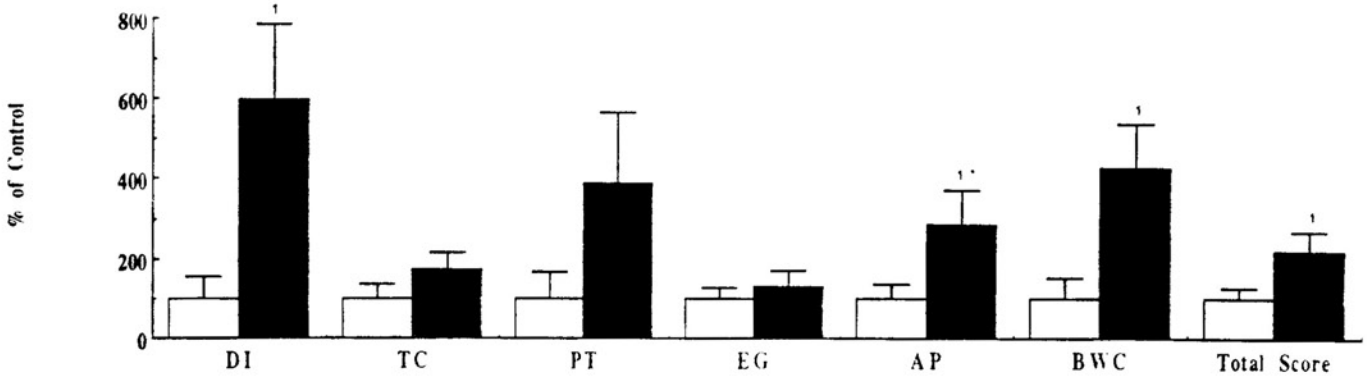
1 - Significant differences from Control group, $p<0.05$.

Figure 5b. Morphine: Percent of preference change from baseline during stress:
History of exposure to stress by current exposure to stress



Abbreviations: C-Control; S-Stress; PC-Partial Conditioning;
CI-Conditioned Inhibition; PS-Paired Stress.

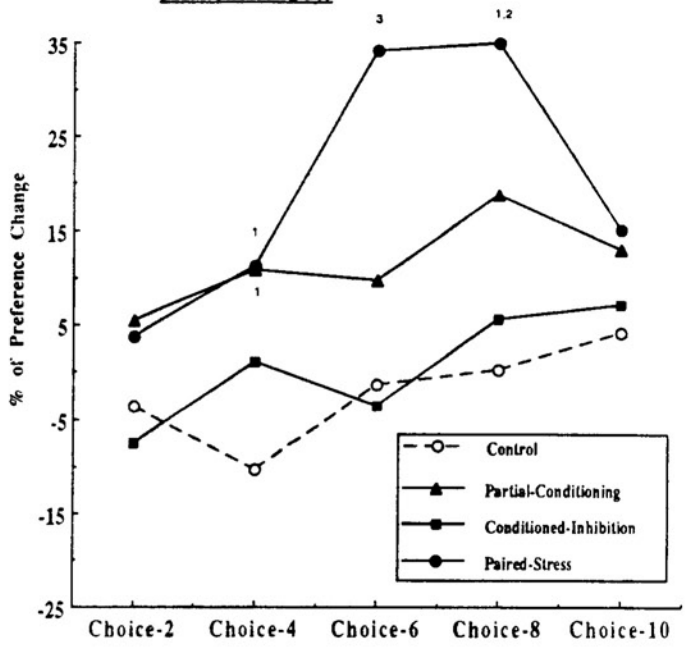
Figure 5c. Morphine withdrawal syndrome - Relapse phase



1 - Significant differences from Control group, $p<0.05$.
1* - Approaching significant differences from Control group, $p<0.1$.

DI - Diarrhea; TC - Teeth chattering; PT - Ptosis; EG - Excessive grooming;
AP - Abnormal posture; BWC - Body weight change.

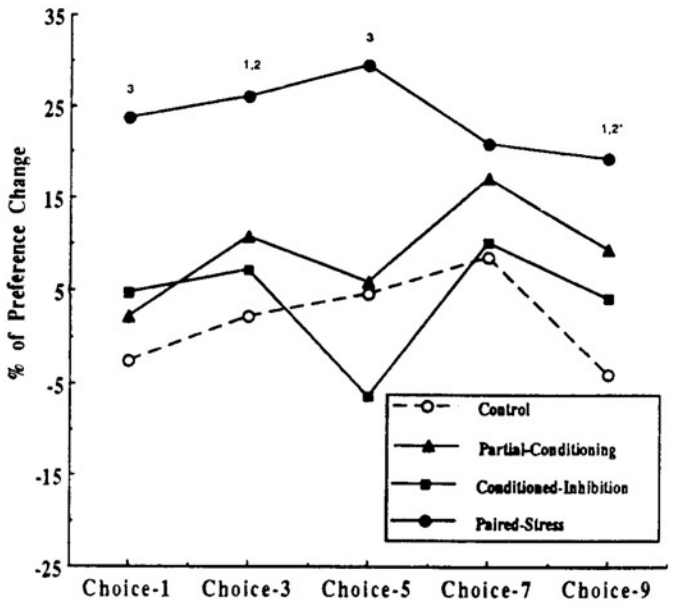
Figure 6a. Fentanyl: Percent of preference change from baseline: *Test Choice Days*



1 - Significant differences from Control group, p<0.05.
1* - Approaching significant differences from Control group, p<0.1.

2 - Significant differences from Conditioned-Inhibition group, p<0.05.
2* - Approaching significant differences from Conditioned-Inhibition group, p<0.1.
3 - Significant differences between Paired-Stress group and the rest of the groups, p<0.05.

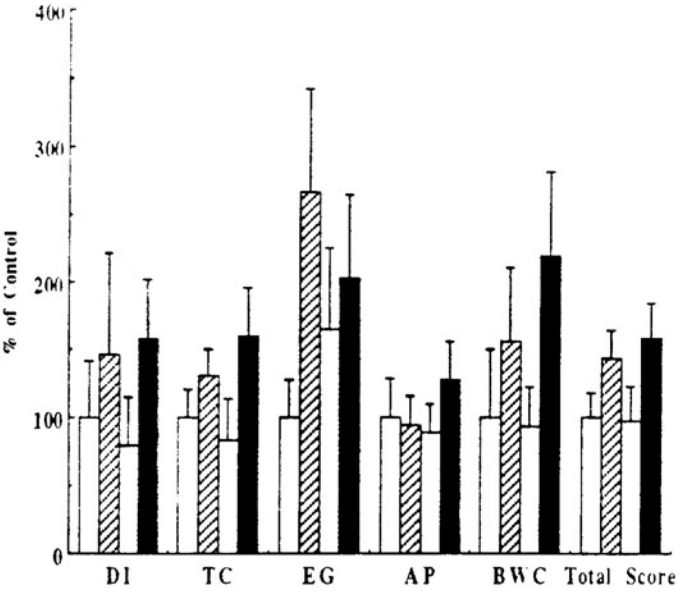
Figure 6b. Fentanyl: Percent of preference change from baseline: *Non-Test Choice Days*



1 - Significant differences from Control group, p<0.05.
1* - Approaching significant differences from Control group, p<0.1.

2 - Significant differences from Conditioned-Inhibition group, p<0.05.
2* - Approaching significant differences from Conditioned-Inhibition group, p<0.1.
3 - Significant differences between Paired-Stress group and the rest of the groups, p<0.05.

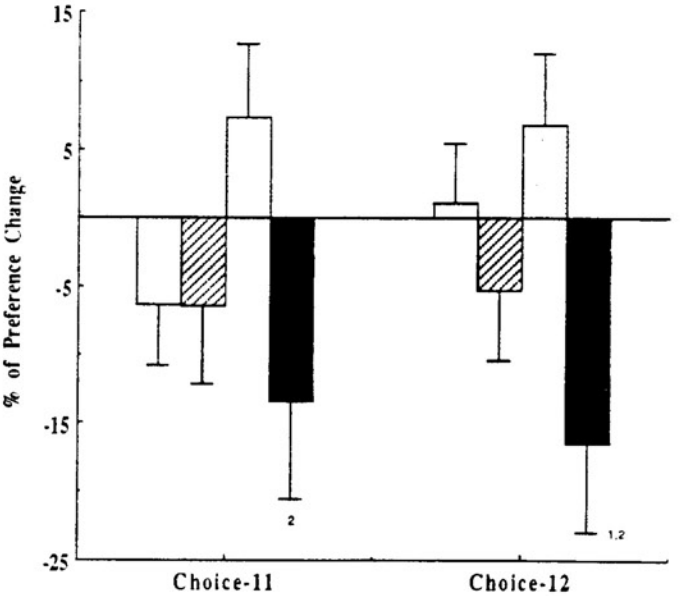
Figure 6c. Fentanyl withdrawal symptoms



1 - Significant differences from Control group, p<0.05.
2 - Significant differences from Conditioned-Inhibition group, p<0.05.

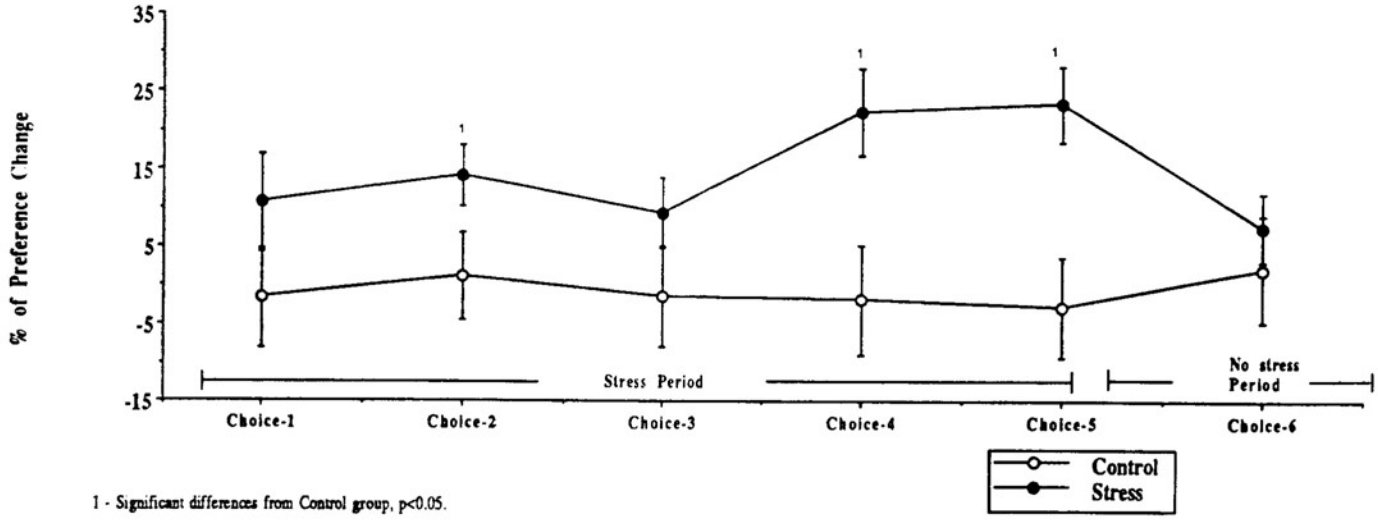
DI - Diarrhea TC - Teeth chattering EG - Excessive grooming
AP - Abnormal posture BWC - Body weight change

Figure 6d. Fentanyl: Percent of preference change from stress period: Phase 2 (no exposure to stress)



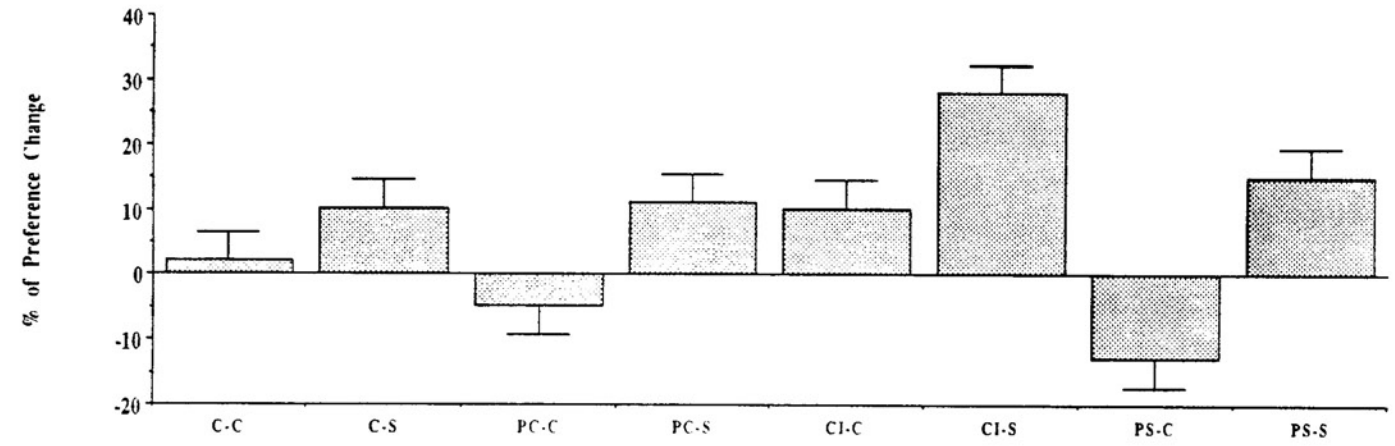
1 - Significant differences from Control group, p<0.05.
2 - Significant differences from Conditioned-Inhibition group, p<0.05

Figure 7a. Fentanyl: Percent of preference change from baseline: Relapse phase



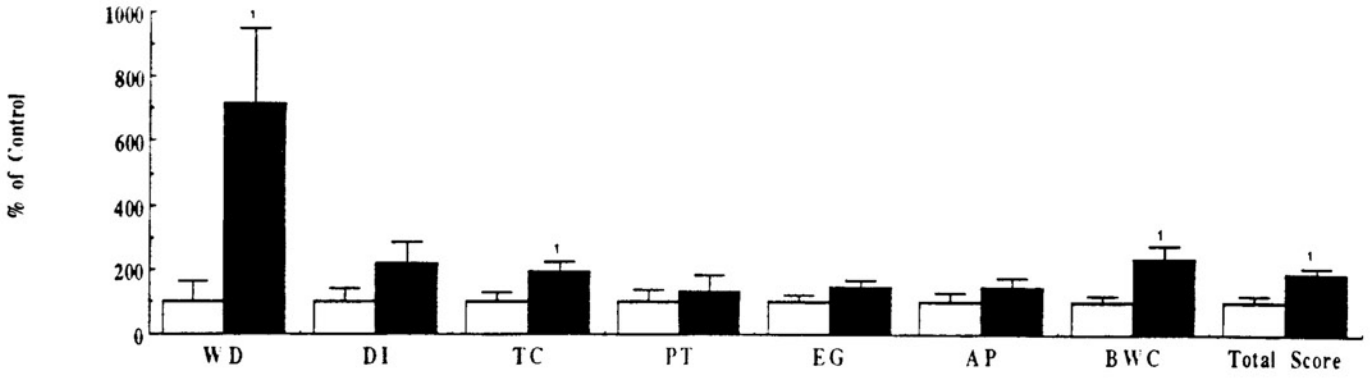
1 - Significant differences from Control group, p<0.05.

Figure 7b. Fentanyl: Percent of preference change from baseline during stress: History of exposure to stress by current exposure to stress



Abbreviations: C-Control; S-Stress; PC-Partial Conditioning; CI-Conditioned Inhibition; PS-Paired-Stress

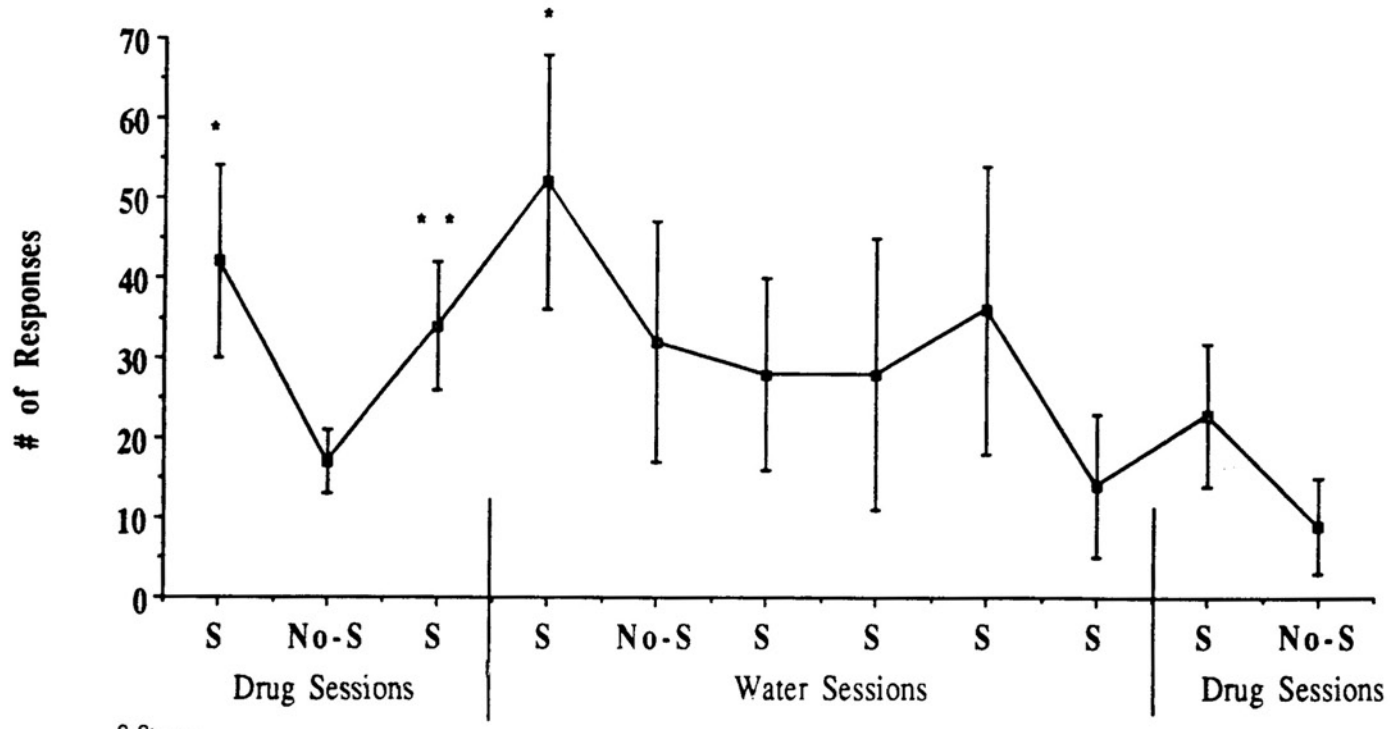
Figure 7c. Fentanyl withdrawal syndrome - Relapse phase



1 - Significant differences from Control group, p<0.05.

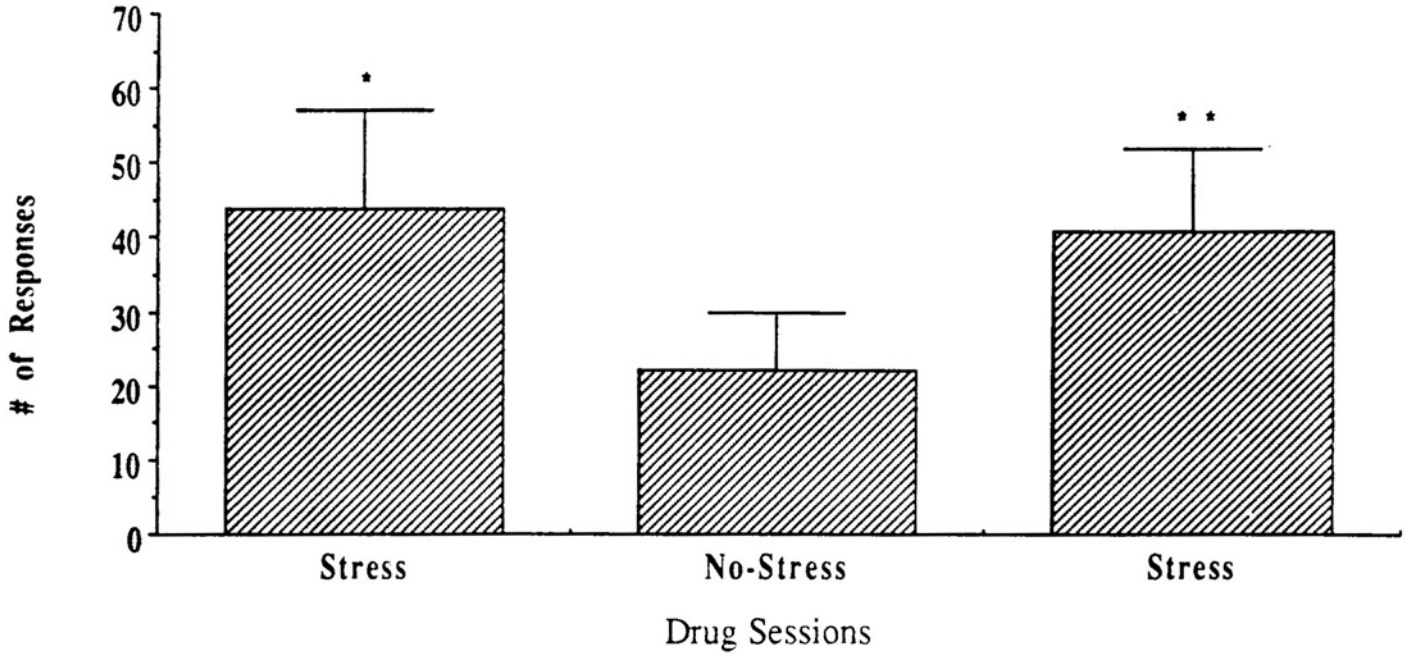
WD - Wet dog shakes; DI - Diarrhea; TC - Teeth chattering; PT - Ptosis; EG - Excessive grooming; AP - Abnormal posture; BWC - Body weight change

Figure 8a. Average number of responses per session: Fixed-ratio-4
(3-day blocks)



S-Stress;.
* - Approaching significant differences from the first No-Stress drug session, $p<0.07$.
** - Significant differences from the first No-Stress drug session, $p<0.05$.

Figure 8b. Average number of responses per session: Progressive-ratio



* - Approaching significant differences from the No-Stress condition, $p<0.07$.
** - Significant differences from the No-Stress condition, $p<0.05$.